

JC03 Rec'd PCT/PTO 10 JAN 2001

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>NIELSEN 4</b>
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/743414</b>
INTERNATIONAL APPLICATION NO. <b>PCT/DK99/00397</b>	INTERNATIONAL FILING DATE <b>12 July 1999</b>	PRIORITY CLAIMED <b>10 July 1998</b>
TITLE OF INVENTION <b>METABOLICALLY ENGINEERED MICROBIAL CELL WITH UNALTERED METABOLITE PRODUCTION</b>		
APPLICANT(S) FOR DO/EO/US <b>Jens NIELSEN et al.</b>		
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <li><input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li><input checked="" type="checkbox"/> The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31).</li> <li><input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li><input type="checkbox"/> is attached hereto (required only if not transmitted by the International Bureau).</li> <li><input type="checkbox"/> has been communicated by the International Bureau.</li> <li><input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</li> <li><input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li><input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li><input type="checkbox"/> have been communicated by the International Bureau.</li> <li><input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li><input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li><input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li><input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol> <p><b>Items 11. to 16. below concern document(s) or information included:</b></p> <ol style="list-style-type: none"> <li><input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li><input type="checkbox"/> An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li><input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</li> <li><input checked="" type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</li> <li><input type="checkbox"/> A substitute specification.</li> <li><input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li><input checked="" type="checkbox"/> Other items or information:             <ul style="list-style-type: none"> <li><input checked="" type="checkbox"/> Courtesy copy of the International Application as filed.</li> <li><input checked="" type="checkbox"/> Courtesy copy of the first page of the International Publication (WO 00/03020).</li> <li><input checked="" type="checkbox"/> <b>Courtesy copy of the International Preliminary Examination Report with annexes containing claims 1-72 to be substituted for the original claims for examination in this case.</b></li> <li><input checked="" type="checkbox"/> Formal drawings, 13 sheets, Figures 1-13.</li> <li><input checked="" type="checkbox"/> Courtesy Copy of the International Search Report.</li> <li><input checked="" type="checkbox"/> Sequence Listing</li> </ul> </li> </ol>		

09/743414

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)  
PCT/DK99/00397

Attorney's Docket No.  
NIELSEN 4

17. [xx] The following fees are submitted:

**BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) - (5):**

Neither international preliminary examination fee (37 CFR 1.482)  
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  
and International Search Report not prepared by the EPO or JPO.....\$1000.00

International preliminary examination fee (37 CFR 1.482) not paid to  
USPTO but International Search Report prepared by the EPO or JPO.....\$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but  
international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$710.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)  
but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)  
and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

Surcharge of \$130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30  
months from the earliest claimed priority date (37 CFR 1.492(e)).

Claims as Originally Presented	Number Filed	Number Extra	Rate
Total Claims	1 - 20		X \$18.00
Independent Claims	1 - 3		X \$80.00
Multiple Dependent Claims (if applicable)			+\$270.00

**TOTAL OF ABOVE CALCULATIONS =**

Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate
Total Claims	20 - 20		X \$18.00
Independent Claims	1 - 3		X \$78.00

**TOTAL OF ABOVE CALCULATIONS =**

Reduction of ½ for filing by small entity, if applicable. Applicant claims small entity  
status. See 37 CFR 1.27.

**SUBTOTAL =**

Processing fee of \$130.00 for furnishing the English translation later than [ ] 20 [ ] 30  
months from the earliest claimed priority date (37 CFR 1.492(f)).

**TOTAL NATIONAL FEE =**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be  
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

**TOTAL FEES ENCLOSED =**

Amount to be:	\$
refunded	
charged	\$

a. [ ] A check in the amount of \$\_\_\_\_\_ to cover the above fees is enclosed.

b. [X] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of \$\_\_\_\_\_, is attached.

c. [ ] Please charge my Deposit Account No. 02-4035 in the amount of \$\_\_\_\_\_ to cover the above fees.  
A duplicate copy of this sheet is enclosed.

d. [XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment  
to Deposit Account No. 02-4035. A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or  
(b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

**BROWDY AND NEIMARK, P.L.L.C.**  
624 NINTH STREET, N.W., SUITE 300  
WASHINGTON, D.C. 20001  
TEL: (202) 628-5197  
FAX: (202) 737-3528

Date of this submission: **January 10, 2001**  
Form PTO-1390 (as slightly revised by Browdy and Neimark)

SIGNATURE

Iver P. Cooper

NAME

28,005

REGISTRATION NUMBER

Page 2 of 2

1097434109/743414  
526 Rec'd PCT/PTO 10 JAN 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit:
Jens NIELSEN et al.	)	
	)	
	)	
IA No.: PCT/DK99/00397	)	
	)	Washington, D.C.
IA Filed: 12 July 1999	)	
	)	
U.S. App. No.:	)	
(Not Yet Assigned)	)	
	)	January 10, 2001
National Filing Date:	)	
(Not Yet Received)	)	
	)	
For: METABOLICALLY ENGINEERED...	)	Docket No.: NIELSEN 4

PRELIMINARY AMENDMENT

Honorable Commissioner for Patents and Trademarks  
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and  
prior to calculation of the filing fee, kindly amend as  
follows:

IN THE SPECIFICATION

After the title please insert the following  
paragraph:

--The present application is the national stage  
under 35 U.S.C. 371 of PCT/FL99/00397, filed 12 July 1999.--

IN THE CLAIMS

Delete claims 2-72.

REMARKS

The above amendment to the specification is being made to insert reference to the PCT application of which the present case is a U.S. national stage. The above amendments to the claims are being made in order to eliminate claims, for the purpose of reducing the filing fee. Please enter this amendment prior to calculation of the filing fee in this case.

Favorable consideration and allowance are earnestly solicited.

Respectfully submitted,  
BROWDY AND NEIMARK, P.L.L.C.  
Attorneys for Applicant

By: 

Iver P. Cooper  
Registration No. 28,005

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Telephone No.: (202) 628-5197  
Facsimile No.: (202) 737-3528

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit:
Jens NIELSEN et al.	)	
	)	
	)	
IA No.: PCT/DK99/00397	)	
	)	Washington, D.C.
IA Filed: 12 July 1999	)	
	)	
U.S. App. No.:	)	
(Not Yet Assigned)	)	
	)	January 10, 2001
National Filing Date:	)	
(Not Yet Received)	)	
	)	
For: METABOLICALLY ENGINEERED...	)	Docket No.: NIELSEN 4

SUPPLEMENTAL PRELIMINARY AMENDMENT

Honorable Commissioner for Patents and Trademarks  
Washington, D.C. 20231

Sir:

Prior to examination upon the merits, kindly amend  
as follows:

Delete claim 1, and insert the following claims:

--73. Recombinant microbial cell comprising

i) a first enzyme activity controlling  
assimilation of a nitrogen nutrient source,

wherein the first enzyme activity is encoded  
by a first nucleic acid operable linked to an  
expression signal not natively associated with  
the first nucleic acid, and

wherein the expression of the first enzyme  
activity is increased as compared to the  
expression of the first enzyme activity when

the first nucleic acid is associated with its native expression signal,

and/or

- ii) a second enzyme activity controlling assimilation of a nitrogen nutrient source,

wherein the second enzyme activity is encoded by a second nucleic acid operable linked to an expression signal not natively associated with the second nucleic acid, and

wherein the expression of the second enzyme activity is increased as compared to the expression of the second enzyme activity when the second nucleic acid is associated with its native expression signal,

the cell further comprising

- iii) a reduced or eliminated expression of a third enzyme activity encoded by a third nucleic acid and controlling assimilation in the cell of a nitrogen nutrient source, wherein the expression of the third enzyme activity is reduced or eliminated as compared to the expression of the third enzyme activity when the third nucleic acid is associated with its native expression signal.

74. Microbial cell according to claim 73, the cell comprising

- i) a further enzyme activity, the further enzyme activity mediates an energy yielding first



83. Microbial cell according to claim 82 wherein the activity is a *Saccharomyces cerevisiae* glutamine synthetase activity, or a functionally equivalent activity capable of catalysing a glutamine synthetase reaction.

84. Microbial cell according to claim 73 wherein the third enzyme activity is a glutamate dehydrogenase activity.

85. Microbial cell according to claim 84 wherein the activity is a *Saccharomyces cerevisiae* glutamate dehydrogenase activity, or a functionally equivalent activity capable of catalysing a glutamate dehydrogenase reaction.

86. Microbial cell according to claim 73, wherein the increased expression of the first and/or second enzyme activity encoded by the first and/or second nucleic acid, respectively, results in an increased production of a first metabolite, the production being increased as compared to the production of the metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.

87. Microbial cell according to claim 86 wherein the first metabolite is ethanol.

88. Microbial cell according to claim 86, wherein the yeast cell further produces a second metabolite, the production of the second metabolite being decreased as compared to the production of the metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.

89. Microbial cell according to claim 88, wherein the second metabolite is glycerol.



90. Method of producing a first metabolite, the method comprising the steps of

- i) cultivating the microbial cell according to claim 1 in a suitable growth medium and under such conditions that the microbial cell is producing a first metabolite, and optionally
- ii) isolating the first metabolite in a suitable form, and further optionally
- iii) purifying the isolated first metabolite.

91. Method of claim 90, wherein the production of the first metabolite is increased in a cell wherein the expression of the first and/or second enzyme activity encoded by the first and/or second nucleic acid, respectively, is increased, as compared to the production of the first metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.

92. Method of claim 90, wherein the yeast cell further produces a second metabolite, the production of the second metabolite is decreased as compared to the production of the second metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.

#### REMARKS

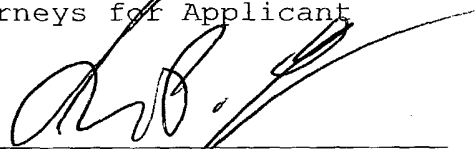
Claims 73-92 presently appear in this case. The above amendments to the claims are being made in order to add new claims and to restore at least partly the varying

scope of claims which was eliminated by the elimination of multiple dependencies in the claims.

Favorable consideration is earnestly solicited.

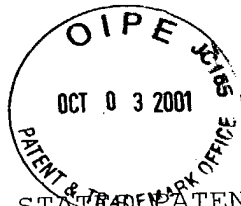
Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.  
Attorneys for Applicant

By:   
Iver P. Cooper  
Registration No. 28,005

IPC:wrd  
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Facsimile No.: (202) 737-3528

09743414  
REC'D PCT/PTO 03 OCT 2001 #6



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Box PCT
	)	
NIELSEN et al	)	Examiner:
	)	
Appln. No.: 09/743,414	)	Washington, D.C.
	)	
IA No. PCT/DK99/00397	)	
IA Filed: July 12, 1999	)	October 3, 2001
	)	
For: METABOLICALLY ENGINEERED	)	Atty.Docket:
MICROBIAL CELL WITH AN	)	NIELSEN=4
ALTERED METABOLITE	)	
PRODUCTION	)	

**RESPONSE TO NOTIFICATION TO COMPLY WITH  
"SEQUENCE LISTING" REQUIREMENT**

Honorable Commissioner of Patents  
Washington, D.C. 20231

Sir:

In response to the Notification to Comply included  
in the Notification of a Defective Response, mailed September  
20, 2001, please amend the application as follows:

IN THE SEQUENCE LISTING

Please substitute the attached Sequence Listing,  
numbered as pages 1-7 for the Sequence Listing previously  
submitted.

Application No. 09/743,414



REMARKS

1. Applicants hereby submit the following:
  - [ ] a paper copy of a "Sequence Listing", complying with §1.821(c), to be incorporated into the specification as directed above;
  - [XX] an amendment to the paper copy of the "Sequence Listing" submitted on June 14, 2001, the amendment being in the form of substitute sheets. Applicants made the corrections as noted in the "Raw Sequence Listing Error Summary".
  - [XX] the Sequence Listing in computer readable form, complying with §1.821(e) and §1.824, including, if an amendment to the paper copy is submitted, all previously submitted data with the amendment incorporated therein;
  - [ ] pursuant to §1.821(e), reference is made to the computer readable form filed on , in USSN , which presents the identical Sequence information, the use of which is now requested, in lieu of submitting a new computer readable form; and/or
  - [ ] a substitute computer readable form to replace one found to be damaged or unreadable.

[ ] 2. The description has been amended to comply with §1.821(d).

3. The undersigned attorney or agent hereby states as follows:

- (a) this submission is not believed to include new matter [§1.821(g)];
- (b) the contents of the paper copy (as amended, if applicable) and the computer readable form of the Sequence Listing, are believed to be the same [§1.821(f) and §1.825(b)];
- (c) if the paper copy has been amended, the amendment is believed to be supported by the specification and is not believed to include new matter [§1.825(a)]; and
- (d) if the computer readable form submitted herewith is a substitute for a form found upon receipt by the PTO to be damaged or unreadable, that the substitute data is believed to be identical to that originally filed [§1.825(d)].

4. Under U.S. rules, each sequence must be classified in <213> as an "Artificial Sequence", a sequence of

Application No. 09/743,414

"Unknown" origin, or a sequence originating in a particular organism, identified by its scientific name.

Neither the rules nor the MPEP clarify the nature of the relationship which must exist between a listed sequence and an organism for that organism to be identified as the origin of the sequence under <213>.

Hence, counsel may choose to identify a listed sequence as associated with a particular organism even though that sequence does not occur in nature by itself in that organism (it may be, e.g., an epitopic fragment of a naturally occurring protein, or a cDNA of a naturally occurring mRNA, or even a substitution mutant of a naturally occurring sequence). Hence, the identification of an organism in <213> should not be construed as an admission that the sequence *per se* occurs in nature in said organism.

Similarly, designation of a sequence as "artificial" should not be construed as a representation that the sequence has no association with any organism. For example, a primer or probe may be designated as "artificial" even though it is necessarily complementary to some target sequence, which may occur in nature. Or an "artificial" sequence may be a substitution mutant of a natural sequence, or a chimera of two or more natural sequences, or a cDNA (i.e., intron-free sequence) corresponding to an intron-containing gene, or otherwise a fragment of a natural sequence.

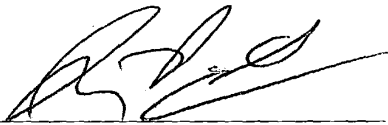
Application No. 09/743,414

The Examiner should be able to judge the relationship of the enumerated sequences to natural sequences by giving full consideration to the specification, the art cited therein, any further art cited in an IDS, and the results of his or her sequence search against a database containing known natural sequences.

Respectfully submitted,

BROWDY AND NEIMARK  
Attorneys for Applicant(s)

By:

  
Iver P. Cooper  
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Box PCT
NIELSEN et al	)	Examiner:
Appln. No.: 09/743,414	)	Washington, D.C.
Filed: January 10, 2001	)	June 14, 2001
For: METABOLICALLY ENGINEERED	)	Atty.Docket: NIELSEN=4
MICROBIAL CELL WITH AN	)	
ALTERED METABOLITE	)	
PRODUCTION	)	

**RESPONSE TO "SEQUENCE LISTING" REQUIREMENT**

Honorable Commissioner for Patents  
Washington, D.C. 20231

Sir:

In response to the Notification to Comply, mailed  
April 30, 2001, please amend the application as follows:

IN THE SPECIFICATION

Please substitute the attached "Sequence Listing"  
for the one previously submitted.

**REMARKS**

1. Applicants hereby submit the following:  
[ ] a paper copy of a "Sequence Listing", complying  
with §1.821(c), to be incorporated into the  
specification as directed above;



[XX] an amendment to the paper copy of the "Sequence Listing" submitted on January 10, 2001, the amendment being in the form of substitute sheets. The only changes made were in the general information sections <130>, <140;141> and <150;151>;

[XX] the Sequence Listing in computer readable form, complying with \$1.821(e) and \$1.824, including, if an amendment to the paper copy is submitted, all previously submitted data with the amendment incorporated therein;

[ ] pursuant to \$1.821(e), reference is made to the computer readable form filed on , in USSN , which presents the identical Sequence information, the use of which is now requested, in lieu of submitting a new computer readable form; and/or

[ ] a substitute computer readable form to replace one found to be damaged or unreadable.

[ ] 2. The description and claims have been amended to comply with §1.821(d).

3. The undersigned attorney or agent hereby states as follows:

(a) this submission is not believed to include new matter [§1.821(g)];

(b) the contents of the paper copy (as amended, if applicable) and the computer readable form of the Sequence Listing, are believed to be the same [§1.821(f) and §1.825(b)];

(c) if the paper copy has been amended, the amendment is believed to be supported by the specification and is not believed to include new matter [§1.825(a)]; and

(d) if the computer readable form submitted herewith is a substitute for a form found upon receipt by the PTO to be damaged or unreadable, that the substitute data is believed to be identical to that originally filed [§1.825(d)].

Application No. 09/743,414

4. Under U.S. rules, each sequence must be classified in <213> as an "Artificial Sequence", a sequence of "Unknown" origin, or a sequence originating in a particular organism, identified by its scientific name.

Neither the rules nor the MPEP clarify the nature of the relationship which must exist between a listed sequence and an organism for that organism to be identified as the origin of the sequence under <213>.

Hence, counsel may choose to identify a listed sequence as associated with a particular organism even though that sequence does not occur in nature by itself in that organism (it may be, e.g., an epitopic fragment of a naturally occurring protein, or a cDNA of a naturally occurring mRNA, or even a substitution mutant of a naturally occurring sequence). Hence, the identification of an organism in <213> should not be construed as an admission that the sequence *per se* occurs in nature in said organism.

Similarly, designation of a sequence as "artificial" should not be construed as a representation that the sequence has no association with any organism. For example, a primer or probe may be designated as "artificial" even though it is necessarily complementary to some target sequence, which may

Application No. 09/743,414

occur in nature. Or an "artificial" sequence may be a substitution mutant of a natural sequence, or a chimera of two or more natural sequences, or a cDNA (i.e., intron-free sequence) corresponding to an intron-containing gene, or otherwise a fragment of a natural sequence.

The Examiner should be able to judge the relationship of the enumerated sequences to natural sequences by giving full consideration to the specification, the art cited therein, any further art cited in an IDS, and the results of his or her sequence search against a database containing known natural sequences.

Respectfully submitted,

BROWDY AND NEIMARK  
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09/743414

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WO 00/03020

PCT/DK99/00397

1

TITLE: Metabolically engineered microbial cell with an altered metabolite production.

#### TECHNICAL FIELD OF THE INVENTION

5 The invention is in the areas of microbial biotechnology, metabolic and genetic engineering. It relates to a microbial cell wherein the expression of a number of expressible enzyme activities have been either increased or decreased or eliminated in order to alter the rate of production and/or the yield of a cellular metabolite such as an intermediate product or an end product of a metabolic pathway.

10

At least one, preferably two or more, of said expressible enzyme activities, when expressed, mediates a reaction involved in the assimilation of a nutrient source in said cell. Assimilation of a nutrient source may involve uptake of said source into the cell and/or conversion of said source within said cell, preferably the incorporation of said source into a biosynthetic product of said cell, said incorporation being controlled by the metabolic potential and capability of said cell.

15

The invention described herein below relates to a manipulation of a process of assimilation of a nutrient source, preferably ammonia, and the resulting effect thereof on the capability of a microbial cell to produce one or more primary and/or secondary metabolites such as intermediate products and/or end products of one or more metabolic pathways present in said microbial cell.

20

#### 25 BACKGROUND OF THE INVENTION

A living cell carries out a complex network of more than a thousand different reactions simultaneously, with each sequence of reactions being strictly and sensitively controlled in a number of ways so that undesirable accumulations or deficiencies of intermediates and/or end products are normally prevented from occurring. As a result of this strict and sensitive control, reactions of great mechanistic complexity and stereochemical selectivity may proceed smoothly under normal physiological conditions such as ambient pressure, moderate temperature and a pH near neutrality.

30

In order to appreciate the complexity and selectivity of the control of metabolic networks, it is necessary first to consider specific reaction sequences such as metabolic pathways; the relationship between each pathway and the cellular architecture; the biological importance of each metabolic pathway; and the sensitive and efficient control mechanisms regulating intracellular reaction rates. The totality of intracellular reaction rates is also known as metabolic flux.

The person skilled in the art will be aware of many microbial primary and secondary metabolites and he will have access to relevant reference collections on the subject such as the authoritative Bergeys Manual. The person skilled in the art will also have to his disposal general biochemistry textbooks comprising state of the art insights into the complex world of cellular metabolism and biochemistry.

Metabolic engineering may be perceived as a purposeful redesigning of metabolic networks generating a change in and/or a redirection of the aerobic and/or the anaerobic metabolism of a microbial cell. State of the art metabolic engineering techniques have been described by among others Cameron and Chaplen (1997) in Curr. Opin. Biotechnol., vol. 8, pages 175 – 180, Hahn-Hägerdal et al. (1996) in Ann. New York Acad. Sci., vol. 782, pages 286 – 296, Stephanopoulos (1994) in Curr. Opin. Biotechnol., vol. 5, pages 196 – 200, Stephanopoulos and Sinskey (1993) in Trends Biotechnol., vol. 11, pages 392 – 396, and Cameron and Tong (1993) in Appl. Biochem. Biotechnol., vol. 38, pages 105 – 140.

Some microbial cells are potentially recognisable by a single characteristic trait in the form of e.g. a metabolic end product predominantly produced under a given set of growth conditions. Accordingly, a yeast may well be initially characterised by a production of ethanol in much the same way as a lactic acid bacterial cell may be potentially identifiable by a production of lactic acid. However, the complex environment wherein microbial cell metabolites are produced evidently leads not only to the formation of a single although predominant metabolite, but rather to a complex set of metabolic intermediates and end products. Many aspects of microbial metabolism and the regulation thereof are still far from being thoroughly understood.

The coenzyme nicotinamide adenine dinucleotide in its oxidised form ( $\text{NAD}^+$ ) contains a nicotinamide ring structure that is readily reducible and thus serves as an oxidising agent. Accordingly, nicotinamide adenine dinucleotide may consist in either a reduced form,  $\text{NADH}$ , or an oxidised form,  $\text{NAD}^+$ . Many dehydrogenase enzymes, such as alcohol dehydrogenases, have a strong affinity for the oxidised form,  $\text{NAD}^+$ . After oxidation of a substrate, the reduced form of the coenzyme,  $\text{NADH}$ , leaves the enzyme and is reoxidised by available electron-acceptor systems in the cell. The  $\text{NAD}^+$  so formed can now bind to another enzyme molecule and repeat the cycle.  $\text{NAD}^+$  and  $\text{NADH}$  differ from most substrates in that they are continually recycled.

5 pathways such as glycolysis, wherein a conversion of glucose results in formation of products such as e.g. ethanol and  $\text{CO}_2$ .

10 mostly obtain free energy released during catabolism in the form of ATP. The chemical energy stored as ATP may be converted to other forms of energy in a process known as energy transduction.

15 and anaerobically growing microbial cells. The major input to glycolysis is glucose and the pathway, comprising a total of 10 different reactions, leads to the conversion of one molecule of glucose to two molecules of pyruvate, with the concomitant generation of ATP as well as the coenzyme NADH.

20 The sequence of reactions between glucose and pyruvate can be considered as two distinct phases, one comprising the first five reactions and constituting an energy input phase, in which sugar phosphates are synthesised at the expense of a conversion of ATP to a less energy rich molecule in the form of ADP, and one phase comprising the last five reactions and representing an energy output phase, in which a transfer of a phosphate group to ADP  
25 leads to regeneration of ATP. The glycolytic conversion of glucose to pyruvate also involves the concomitant reduction of two moles of  $\text{NAD}^+$  to its reduced equivalent NADH.

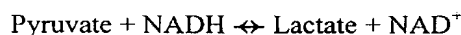
carbon dioxide. Ethanol production through anaerobic fermentation of a carbon source by the yeast *Saccharomyces cerevisiae* is one of the best known biotechnological processes and accounts for a world production of approximately 30 billion litres per year. The ethanol yield is lower than a maximum, theoretical yield due to a formation of a number of additional



products affecting the ethanol yield, such as *e.g.* biomass, acetate, pyruvate, succinate and glycerol. A *de novo* synthesis of the first four components results in a net formation of NADH, while a synthesis of glycerol occurs under simultaneous NADH consumption. As ethanol is synthesised without a net formation or consumption of NADH, glycerol formation plays an important physiological role under anaerobic growth. Glycerol formation leads to a reoxidation of NADH to NAD<sup>+</sup> and thereby substitutes the role of oxygen as an electron acceptor.

It is known that in anaerobic cultivations of *Saccharomyces cerevisiae* CBS8066, approximately 10% of the carbon source is directed towards the formation of glycerol (Nissen *et al.*, 1997; Verduyn *et al.*, 1990). A redirection of this amount of carbon towards ethanol production is clearly desirable and would presuppose a reduction in the net formation of NADH in the synthesis of biomass and organic acids.

Accordingly, for the glycolytic pathway to operate anaerobically, i.e. in the absence of oxygen, NADH must be reoxidised to NAD<sup>+</sup> by means of a transfer of electrons to a suitable electron acceptor so that a steady metabolic flux can be maintained. Microbial cells growing anaerobically have different ways of transferring such electrons. A simple route used by lactic acid bacteria consists of simply using NADH to reduce pyruvate to lactate, via the enzyme lactate dehydrogenase. NADH is reoxidised in the process:



The lactic acid fermentation, i.e. conversion of glucose to lactic acid, is important in the manufacture of cheese. Another important fermentation involves a conversion of pyruvate to acetaldehyde and CO<sub>2</sub> and a reduction of acetaldehyde to ethanol by alcohol dehydrogenase:



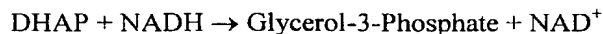
When carried out by yeast cells, this fermentation generates the alcohol in alcoholic beverages. Yeast cells used in baking also carry out this form of fermentation and the CO<sub>2</sub> produced by pyruvate decarboxylation causes bread to rise while the ethanol produced evaporates

during baking. Among many other useful fermentations are those leading to e.g. acetic acid in the manufacture of vinegar and propionic acid in the manufacture of Swiss cheese.

Glycerol formation in cellular metabolism has at least two physiologically important roles in *Saccharomyces cerevisiae* – it is involved in NADH reoxidation and it acts as an efficient osmolyte that protects the cell against lysis under stress conditions.

Synthesis of biomass and organic acids, *i.e.* succinic acid, acetic acid and pyruvic acid, results in a net formation of intracellular NADH (Oura, 1977; van Dijken & Scheffers, 1986; Nissen *et al.*, 1997). This has to be balanced by a mechanism in which NADH is reoxidised to NAD<sup>+</sup> in order to avoid depletion of the NADH pool. Under anaerobic conditions, NADH reoxidation is not possible by means of the respiratory chain, which is not functioning under such conditions. Instead, NADH is reoxidised to NAD<sup>+</sup> via formation of glycerol, since synthesis of one molecule of glycerol from glucose leads to reoxidation of one molecule of NADH.

Glycerol is also formed and accumulated inside the cell during growth under osmotic stress conditions and acts as an efficient osmolyte that protects the cell against lysis (Ansell *et al.*, 1997; Larsson *et al.* (1993)). The formation of glycerol occurs via a two step reaction from dihydroxyacetone phosphate (DHAP) that is catalysed by glycerol 3-phosphate dehydrogenase and glycerol 3-phosphate phosphatase, respectively.



In order to be able to produce any metabolic product, the microbial cell needs an input in the form of both energy and readily assimilable nutrient sources. The metabolism of a microbial cell very much determines the capability of said cell to exploit nutrient sources present in an external environment. Consequently, the metabolism of a microbial cell is dynamic and the sensitive regulation, direction and redirection of said metabolism is indicative of the responses of said cell to changing environmental conditions.

Assimilable nutrients such as various sources of nitrogen, carbon, sulphur and phosphor exist in many different forms. Some of these forms may be readily assimilated by a microbial cell while others cannot be assimilated. In the case of nitrogen, it is essential that a microbial cell is capable of assimilating this nutrient source, as nitrogen forms part of among  
5 others i) amino acids in proteins, ii) nucleotides in DNA and RNA, iii) amino sugars in complex polysaccharides, and iv) heterocyclic compounds in various coenzymes.

As described above, catabolic and anabolic pathways occur in different stages of complexity and one of said stages involves the ultimate degradation to, or synthesis from, inorganic compounds such as  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , and  $\text{NH}_3$ . The majority if not all microbial cells are capable of assimilating ammonia and converting this source of nitrogen into organic nitrogen compounds - i.e. any organic compound comprising a C-N bond. Ammonia is thus a central metabolite and actually serves as a substrate for no less than five different enzymes that convert it into various organic nitrogen-comprising compounds. At physiological pH, the dominant ionic species is an ammonium ion, but all of said five reactions involve the unshared electron pair of  $\text{NH}_3$ , which is therefore generally considered the reactive species.

Accordingly, microbial cells assimilate ammonia via reactions leading to the formation of either glutamate, glutamine, asparagine, or carbamoyl phosphate. Because carbamoyl phosphate is used only in the biosynthesis of arginine, urea, and the pyrimidine nucleotides, most of the nitrogen ending up in amino acids and other nitrogen comprising organic compounds is assimilated via the two amino acids glutamate and glutamine. The enzymes responsible for ammonia assimilation in a microbial cell are briefly introduced herein below.

25 Glutamate dehydrogenase catalyses the reductive amination of 2-oxoglutarate:



Microbial cells growing with ammonia as their sole nitrogen source use the above reaction  
30 as a primary route for nitrogen assimilation.

Most microbial cells contain an NADPH-specific form of the glutamate dehydrogenase enzyme, as indicated above, which acts primarily in the direction of glutamate formation. Inte-

restingly, yeast contain both a NADH-specific form and a NADPH-specific form of the enzyme, each form being appropriately regulated, with one form, NADPH, primarily involved in nitrogen assimilation and the other, NADH, functioning primarily in catabolic metabolism.

5

The major source of electrons for reductive biosynthesis is NADPH, nicotinamide adenine dinucleotide phosphate.  $\text{NADP}^+$  and NADPH are identical to  $\text{NAD}^+$  and NADH, respectively, except that the form has an additional phosphate esterified at C-2' on the adenylate moiety.  $\text{NAD}^+$  and  $\text{NADP}^+$  are equivalent in their thermodynamic tendency to accept electrons and they have similar standard reduction potentials. For reasons not known, nicotinamide nucleotide-linked enzymes that act in catabolic metabolism usually use the  $\text{NAD}^+/\text{NADH}$  coenzyme pair, whereas those acting in anabolic pathways tend to use  $\text{NADP}^+/\text{NADPH}$ .

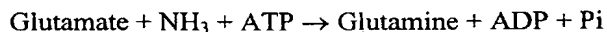
15 Glutamate synthase is an enzyme functionally related to glutamate dehydrogenase and catalyses a reaction comparable to that catalysed by glutamate dehydrogenase. However, glutamate synthase functions primarily in glutamate biosynthesis:



20

When formed by the action of glutamate dehydrogenase, glutamate can accept a second ammonia moiety to form glutamine in a reaction catalysed by the enzyme glutamine synthetase:

25



This enzyme is named a synthetase, rather than a synthase, because the reaction couples bond formation with the energy released from ATP hydrolysis. However, both enzymes are classified as ligases, but a synthase enzyme does not require ATP.

30

Glutamine synthetase of *E. coli* is a dodecamer, whose 12 identical subunits form two facing hexagonal arrays. The holoenzyme has a molecular weight of about 600,000. Each ca-

talytic site is formed at an interface between polypeptide subunits within a hexamer and is made up of residues from two adjacent subunits.

Glutamine occupies a central role in the nitrogen metabolism of any microbial cell. The  
5 amide nitrogen is used in biosynthesis of several amino acids, including glutamate, tryptophan, and histidine, purine and pyrimidine nucleotides, and amino sugars. As revealed primarily based on studies in *E. coli*, several remarkable and quite extraordinary control mechanisms for glutamine synthetase mediated reactions interact with one another in very complex ways. The activity of glutamine synthetase is controlled by two distinct but mutu-  
10 ally related mechanisms: Allosteric regulation by cumulative feedback inhibition and covalent modification of the enzyme mediated by a regulatory cascade.

Cumulative feedback inhibition involves the action of no less than eight specific feedback inhibitors. Those eight inhibitors are either metabolic end products of glutamine metabolism  
15 (tryptophan, histidine, glucosamine-6-phosphate, carbamoyl phosphate, CTP, and AMP), or they are indicators in various ways of the general status of amino acid metabolism (alanine, glycine). Quite remarkably, each 50,000-dalton subunit of glutamine synthetase contains binding sites for each of the eight inhibitors, as well as binding sites for substrates and products.

20 Each of the eight compounds alone gives only partial inhibition, but in combination the degree of inhibition is increased until a mixture of all eight provides a virtually complete blockage. This ensures that an accumulation of an end product of one pathway does not shut off the supply of glutamine needed for another pathway. Glutamine synthetase is also regulated by means of adenylation. An enzyme molecule with all 12 sites adenylylated is completely inactive, whereas partial adenylylation yields a correspondingly partial inactivation.  
25

Adenylation and deadenylation of glutamine synthetase involve a complex series of regulatory cascades. These regulatory cascades provide a responsive mechanism ensuring  
30 that, when the supply of activated nitrogen in the form of glutamine is sufficiently high, its further biosynthesis is shut down. In contrast, when activated nitrogen in the form of glutamine is low, 2-oxoglutarate accumulates and, provided that ATP is also abundant, stimulates the activity of glutamine synthetase by the converse mechanism.

EP 0 785 275 A2 discloses a yeast comprising constitutive expression of a gene encoding an enzyme activity involved in hexose transport.

EP 0 645 094 A1 discloses the use of a yeast comprising a glycolytic pathway comprising a futile cycle generated by means of a constitutive expression of genes encoding fructose-1,6-biphosphatase and phosphoenolpyruvate carboxykinase.

- 5 US 5,545,556 discloses a yeast strain having a reduced or increased production of glycerol mediated by mutations in various gene-encoded products.

None of the above disclose a microbial cell wherein the expression of a number of expressible enzyme activities involved in nutrient assimilation are either increased or decreased or  
10 eliminated in order to alter the rate of production and/or the yield of a cellular metabolite such as an intermediate product or an end product of a metabolic pathway.

#### SUMMARY OF THE INVENTION

15

It has now surprisingly been discovered that it is possible to operably link the process of nutrient assimilation in a microbial cell such as e.g. a yeast cell with an increased production of a metabolite like e.g. ethanol. The invention is based on the unexpected and surprising finding that it is possible to operably link an energy requiring reaction such as assimilation  
20 of a nutrient source with an energy yielding reaction such as the formation of a desirable metabolic product like e.g. ethanol.

When the nutrient source is a nitrogen source such as ammonia or a nitrogen source convertible into ammonia, it is preferred to substantially reduce or eliminate the expression in a  
25 microbial cell of an enzyme, glutamate dehydrogenase, which, under normal physiological conditions, is the predominant enzyme activity involved in ammonia assimilation, and concomitantly with said reduction or elimination, increase the expression of one or both of two additional enzyme activities, glutamate synthase and glutamine synthetase, both of which are capable of assimilating ammonia into glutamate under the consumption of ATP and re-  
30 oxidation of NADH. Accordingly, overexpression of glutamate synthase and glutamine synthetase in a microbial cell, preferably a yeast cell, under anaerobic conditions, generates a reduction of intracellular ATP levels and potentially a depletion of the pool of ATP available to the cell. Under anaerobic conditions, the cell is capable of counteracting the reduc-

tion in the ATP pool by producing an increased amount of a metabolite such as e.g. ethanol via an ATP yielding reaction.

The world ethanol production reached an estimated 31.3 billion litres in 1996. Approximately 80% were produced by anaerobic fermentation of various sugar sources by *Saccharomyces cerevisiae*. Accordingly, ethanol is one of the most important biotechnological products with respect to both value and amount. Two thirds of the production is located in Brazil and in the United States with the primary objective of using ethanol as a renewable source of fuel. The demand and growth of this market is expected to give rise to a substantial growth in the ethanol production industry in the future. Hence, there are strong economic incentives to further improve the ethanol production process.

The price of the sugar source is a very important process parameter in determining the overall economy of ethanol production. Hence, it is of great interest to optimise the ethanol yield in order to ensure an efficient utilisation of the carbon source. Besides biomass and carbon dioxide, a number of by-products are formed during an anaerobic fermentation of *Saccharomyces cerevisiae* (Oura, 1977). Glycerol is the most dominant of these compounds, consuming up to 4% of the carbon source in industrial fermentations. Accordingly, it is highly desirable to eliminate formation of this compound, when it is not wanted, and redirect the metabolic flux towards ethanol production. If successfully achieved, it should in theory be possible to increase the ethanol yield by a maximum of 4%, corresponding to an increase in the world production of ethanol of 1.25 billion litres per year without any additional costs.

25 Although it is extremely difficult to alter or redirect a microbial metabolism, such as an anaerobic yeast metabolism, it may never the less be desirable to alter a "traditional" profile of primary and/or secondary metabolites in order to achieve a different composition or "product mix", or in order to increase or decrease or even eliminate the production of some metabolites present in said profile.

30 The surprising and unexpected finding described in the present invention makes it possible to i) manipulate the process of assimilation of a nutrient source such as ammonia into or in a microbial cell and ii) correlate said manipulation of said process with a provision of an altered profile of produced metabolites including e.g. ethanol and glycerol.



The correlation of assimilation of a nutrient source with primary and/or secondary metabolite production is achieved by bridging said assimilation with an altered production of a metabolite - an intermediate product or an end product of a metabolic pathway - through an extremely complex and not thoroughly understood network of metabolic reactions guiding the flux of metabolites in a microbial cell.

Accordingly, in a first aspect of the invention there is provided a microbial cell comprising

i) a first expressible enzyme activity which, when expressed in said microbial cell, is controlling assimilation in said cell of a nutrient source, said expression of said first enzyme activity in said microbial cell being either novel or altered as compared to the expression of said first enzyme activity in a comparable wild-type microbial cell or a comparable isolated microbial cell,

and optionally

ii) a second expressible enzyme activity which, when expressed in said microbial cell, is controlling assimilation in said cell of a nutrient source, said expression of said second enzyme activity in said microbial cell being either novel or altered as compared to the expression of said second enzyme activity in a comparable wild-type microbial cell or a comparable isolated microbial cell, said second expressible enzyme activity being non-identical to said first expressible enzyme activity,

and

iii) a reduced expression or no expression of a third expressible enzyme activity which, when expressed in said microbial cell, is controlling assimilation in said cell of a nutrient source, said third expressible enzyme activity being non-identical to any and both of said first and second expressible enzyme activities,

and further optionally

5

10

20

25

30

and optionally

ii) isolating said first metabolite in a suitable form,

and further optionally

iii) further purifying said isolated first metabolite.

10 In an even further aspect there is provided a method of constructing a microbial cell according to the invention, said method comprising the steps of

i) operably linking a nucleotide sequence encoding said first expressible enzyme activity with an expression signal not natively associated with said nucleotide sequence, and/or

ii) operably linking a nucleotide sequence encoding said second expressible enzyme activity with an expression signal not natively associated with said nucleotide sequence, and

iii) eliminating said third expressible enzyme activity from said microbial cell, or optionally operably linking a nucleotide sequence encoding said third expressible enzyme activity with an expression signal not natively associated with said nucleotide sequence, said expression signal generating a reduced expression of said nucleotide sequence, and

iv) introducing said operably linked nucleotide sequences obtained under i) and iii), and optionally the nucleotide sequence obtained under ii), into said microbial cell, or

v) introducing said operably linked nucleotide sequence obtained under i), and optionally the nucleotide sequence obtained under ii), into said microbial cell

obtained under iii) wherein said third expressible enzyme activity has been eliminated.

Preferred embodiments of the above-mentioned aspects of the invention are described herein below.

#### DETAILED DESCRIPTION OF THE INVENTION

Attempts have been made to increase ethanol formation in yeast by elimination of glycerol synthesis through deletions of *GPD1* and *GPD2*, encoding the two existing isoenzymes of glycerol 3-phosphate dehydrogenase (Björkqvist et al., 1997). The double deletion mutant is unable to grow under anaerobic conditions due to accumulation of intracellular NADH. NADH is accumulated since no alternative pathways to reoxidise NADH under these growth conditions exist in *S. cerevisiae*. Elimination of the capability of generating glycerol results in a strain with a high sensitivity to osmotic stress. Osmotic stress is caused by growth of a cell in an industrial growth medium high in concentrations of various carbon sources and salts. Deletion of one of the genes results neither in a significant reduction in glycerol formation nor in an increased ethanol formation (Liden *et al.*, 1997; Nissen *et al.*, 1998a,b).

Consequently, metabolic engineering of the synthesis of glycerol has so far not proved successful and no significant increase in ethanol production in a metabolically engineered *S. cerevisiae* strain has been reported.

It has now surprisingly been found that it is possible to implement a strategy comprising a reduction of a surplus of NADH formed by catabolic metabolism concomitantly with an increased consumption of ATP in the synthesis of biomass.

Ammonia is often used as a nitrogen source in industrial fermentations of *S. cerevisiae*. Following transport across the membrane into the cytoplasm, ammonia or the ammonium ion is converted into glutamate by assimilation with 2-oxoglutarate. In wild-type cells this

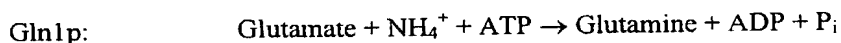
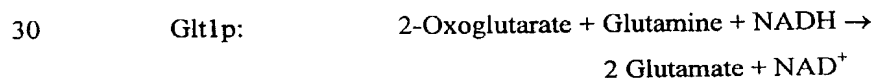
reaction is catalysed by an NADPH-dependent glutamate dehydrogenase encoded by *GDH1* (Moye *et al.*, 1985):

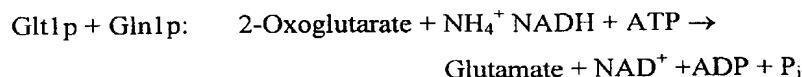


Two other glutamate dehydrogenases, encoded by *GDH2* and *GDH3*, are also present in *S. cerevisiae*. Gdh2p normally catalyses the opposite reaction as that of Gdh1p under formation of NADH (Miller and Magasanik, 1990; Miller and Magasanik, 1991; Coschigano *et al.*, 1991; Courchesne and Magasanik, 1988.). This reaction occurs when nitrogen sources other than ammonium and glutamine are used. The reaction may also play a role in ammination reactions during synthesis of various amino acids.

The activity of Gdh2p is 70 times lower than the activity of Gdh1p, when ammonium is used as nitrogen source (Nissen *et al.*, 1997). It has been demonstrated, as described herein below and in Example 1, that formation of glycerol can be reduced by deleting *GDH1* and overexpressing *GDH2*. These manipulations result in a genetically modified yeast strain synthesising glutamate under consumption of NADH rather than NADPH (Nissen *et al.*, 1998a). However, this reduction in glycerol formation did not result in an overall increase in the ethanol yield. The function of NADPH-dependent Gdh3p is unknown. No activity of a NADPH-dependent glutamate dehydrogenase can be measured in cells with a deletion in *GDH1* when ammonium is used as nitrogen source, suggestion an involvement of this enzyme primarily when other nitrogen sources are used (Nissen *et al.*, 1998a; Avendanö *et al.*, 1997).

Interestingly, there exist another system capable of synthesising glutamate in *S. cerevisiae*. This system consists of two coupled reactions, catalysed by glutamate synthase, encoded by *GLT1*, and glutamine synthetase, encoded by *GLN1* (Cogoni *et al.*, 1995, Miller and Magasanik, 1983). The reactions mediated by said enzymes are briefly illustrated below.





5 In wild-type cells, the activity of Glt1p is 80 times lower than the activity of Gdh1p. Hence, the system can be expected to have a very limited effect on the assimilation of ammonium in a yeast cell.

If *GDH1* is deleted from a yeast cell, the cell becomes unable to assimilate ammonium by  
10 the means of NADPH-dependent glutamate dehydrogenase activity. This leads to a decrease in the maximum specific growth rate to half of that observed for a wild-type yeast cell (Nissen *et al.*, 1998a). This is probably due to a limitation in glutamate synthesis, since other enzymes potentially involved in this process are expressed at very low levels, as described herein above.

15 A double mutant in *GDH1* and *GLT1* is unable to grow on ammonium as nitrogen source, indicating that glutamate synthase potentially could have a role as a backup system in the synthesis of glutamate (Miller and Magasanik, 1990). Thus, it was hypothesised that by overexpressing *GLT1* and *GLN1* in a *Δgdh1* mutant, it should be possible to alleviate and/or  
20 eliminate the limiting effect of glutamate synthesis on  $\mu_{\max}$  and obtain a *S. cerevisiae* strain with a reduced surplus formation of NADH and an increased consumption of ATP in biomass synthesis.

It has been demonstrated that deletion of *GDH1*, encoding the NADPH-dependent glutamate dehydrogenase I, in a wild-type strain of *S. cerevisiae* resulted in an increase in ethanol  
25 formation and a decrease in glycerol formation (Nissen *et al.*, 1998a,b). Unfortunately, the ethanol productivity was significantly affected by the deletion since the maximum specific growth rate of the *Δgdh1* mutant was approximately half of that of the wild-type strain.

30 The present invention has demonstrated for the first time that overexpression of *GLN1*, encoding glutamine synthetase, and *GLT1*, encoding glutamate synthase, in a *Δgdh1* mutant results in a significant increase in the maximum specific growth rate, as compared to the *Δgdh1* mutant, as well as a substantially increased yield of ethanol.

Consequently, a novel pathway for glutamate synthesis has been shown to substitute the role of an NADPH-dependent glutamate dehydrogenase in the assimilation of ammonium and 2-oxoglutarate to glutamate.

5

The maximum specific growth rate of a strain according to the invention, TN19, was 90% of the maximum specific growth rate of the wild-type and this clearly limits the increase in the specific ethanol productivity that was obtained. This problem can most likely be solved by increasing the specific activities of Gln1p and Glt1p even more. In one embodiment of the invention, a five-fold increase in the specific activity of glutamate synthase was obtained by insertion of the *PGK* promoter into chromosome IV in front of the structural gene of the enzyme. This level of activity can be increased significantly by e.g. inserting more copies of the gene into the chromosome by means of e.g. amplification. It is also possible to subject the expression system in question to a detailed genetic and/or biochemical analysis in order to find ways of increasing the expression.

15

In an earlier study, it was demonstrated that overexpression of *GDH2*, encoding the NADH-dependent glutamate dehydrogenase, in a *Agdh1* mutant led to an increase in the maximum specific growth rate from  $0.22 \text{ h}^{-1}$  to  $0.39 \text{ h}^{-1}$  (Nissen *et al.*, 1998a). The specific activity of Gdh2p in this strain was 0.625 units per mg TCP, ten-fold higher than in the wild-type, which illustrates that the activity of Glt1p and Gln1p should be increased further in order to achieve the same maximum specific growth rate as the wild-type.

20

The limited increase in the fermentation time of TN19 compared to TN1 might not be a serious problem, as it is the substrate cost that is a primary factor determining the overall economy of ethanol production. This means that the observed increase in the ethanol yield of TN19, combined with the relative small reduction of the maximum specific growth rate, represents a breakthrough in and a valuable contribution to an optimisation of microbial ethanol production.

25  
30

Furthermore, the results obtained in this study showed that even though it is very difficult to perform a metabolic engineering process, the proposed strategy of increasing the ethanol

yield in *S. cerevisiae* by metabolic engineering of pathways involved in nutrient assimilation and biomass synthesis is a major success.

Accordingly, it has been demonstrated that a mere reduction of glycerol formation via metabolic engineering of NADH- and NADPH-consuming reactions does not necessarily result in an increased flux towards ethanol, and said reduced glycerol formation must accordingly, as convincingly demonstrated herein by the provision of impressive results representing a breakthrough in microbial ethanol production, be combined with an increased consumption of ATP in biomass formation in order to redirect carbon flux from glycerol towards an increased production of ethanol.

The microbial cell according to the invention comprises an altered composition of expressible enzyme activities and/or an altered expression thereof. In principle, any microbial cell capable of i) assimilating a nutrient source, ii) metabolising said source, and iii) producing a biosynthetic product in the form of e.g. one or more primary and/or secondary metabolites, forms part of the invention. The expressible enzyme activities such as a first and/or second expressible enzyme activity are preferably operably linked to an expression signal not natively associated with said activities.

In a preferred embodiment there is provided a microbial cell comprising

i) an increased expression of said first expressible enzyme activity controlling assimilation in said cell of a nutrient source, said first expressible enzyme activity being operably linked to an expression signal not natively associated with said first enzyme activity,

and optionally

ii) an increased expression of said second expressible enzyme controlling assimilation in said cell of a nutrient source, said second expressible enzyme activity being operably linked to an expression signal not natively associated with said second enzyme activity,



and

5                   iii) a reduced expression or no expression of said third expressible enzyme activity which, when expressed in said microbial cell, is controlling assimilation in said cell of a nutrient source, said third expressible enzyme activity being optionally operably linked to an expression signal not natively associated with said third enzyme activity,

and further optionally

10                   iv) a fourth expressible enzyme controlling an intracellular redox system of said cell, said fourth expressible enzyme activity being operably linked to an expression signal not natively associated with said fourth enzyme activity.

15                   In another preferred embodiment, the microbial cell of the invention comprises a further expressible enzyme activity, said further expressible enzyme activity, when expressed, mediates an energy yielding first reaction resulting in a production of a first metabolite, said first reaction being operably linked to an energy requiring second reaction resulting in assimilation of a nutrient source. The microbial cell is preferably one wherein said energy requiring second reaction resulting in assimilation of a nutrient source is controlled at least by  
20                   said first and/or second expressible enzyme activity.

25                   In yet another embodiment there is provided a microbial cell comprising a further expressible enzyme activity, said further expressible enzyme activity, when expressed, mediates an energy yielding first reaction resulting in a production of a first metabolite, said further expressible enzyme activity, when expressed at an increased level, results in an increased production of said first metabolite, said increased expression of said further expressible enzyme activity and/or said increased production of said first metabolite is operably linked to an increased expression of said first and/or second expressible enzyme activity.

30                   The expression of said further expressible enzyme activity preferably results in the production of an intermediate or an end product of a metabolic pathway such as metabolites like e.g. lactic acid, acetic acid, propionic acid or ethanol, or a combination thereof. The energy yielding first reaction may accordingly be mediated by a dehydrogenase enzyme such as e.g.



*lactis subsp. diacetylactis*, *Leuconostoc* species, *Lactobacillus* species, *Pediococcus* species and similar industrially relevant species like e.g. *Bifidobacterium*.

The nutrient source is any nutrient source capable of sustaining microbial growth by e.g. being assimilated into a biosynthetic product that can be utilised by a microbial cell or be converted i.e. metabolised into a further biosynthetic product, said utilisation and/or metabolism involving one or more energy yielding metabolic reactions. Consequently, it will be understood that any nutrient source that is assimilable and metabolisable by a microbial cell forms part of the invention. The step of assimilation shall be understood to comprise both uptake of said source into said cell as well as conversion of said source into a biosynthetic product - an intermediate metabolite - within said cell. In a more narrow scope of the understanding of the term, assimilation shall be meant preferably to comprise the assimilation that takes place within the cell without necessarily being limited to this step of the assimilation process.

In a preferred embodiment of the invention there is provided means for an increased efficiency of an uptake of a nutrient source into a microbial cell and/or an increased efficiency of assimilation within said cell. The term efficiency shall be understood to comprise, that both uptake and/or intracellular conversion takes place at a faster rate, i.e. increased amounts of nutrients are taken up and/or metabolised per unit time, or per unit cell time per unit cell mass, in said cell according to the invention, as compared to a comparable wild-type cell or a comparable isolated cell. The person skilled in the art will be aware as to how a trans-membrane transport and an internal turnover of an assimilated nutrient source may be monitored.

The nutrient source is preferably a nitrogen source, a carbon source, a sulphur source, or a phosphorus source, more preferably a nitrogen source. Preferred assimilable nitrogen sources comprise ammonia, ammonium ions, nitrite ions, and nitrate ions. The assimilable nitrogen source according to the invention is more preferably ammonia and ammonium ions and most preferably ammonia. It will be understood that the invention pertains to all nitrogen containing nutrient sources capable of being converted into ammonia by oxidation including biological oxidation or by reduction including biological reduction. The skilled person will

The microbial cell according to the invention comprises a first expressible enzyme activity which, when expressed in said microbial cell, is controlling assimilation in said cell of a nutrient source, preferably a nitrogen source such as e.g. ammonia, said expression of said first enzyme activity in said microbial cell being either novel or altered as compared to the expression of said first enzyme activity in a comparable wild-type microbial cell or a comparable isolated microbial cell.

In a preferred embodiment, the microbial cell comprises a first and a second expressible enzyme activity which, when expressed in said microbial cell, are controlling assimilation in said cell of a nutrient source, preferably a nitrogen source such as e.g. ammonia, said expression of said first and second enzyme activities in said microbial cell is either novel to said cell or altered as compared to the expression of said first and second enzyme activities in a comparable wild-type microbial cell or a comparable isolated microbial cell, said first and second expressible enzyme activities being non-identical to one another.

An expressible enzyme activity mediated facilitation of assimilation of a nutrient source is understood to comprise the capability of said microorganism to carry out a metabolic reaction leading to assimilation in the form of uptake and/or intracellular conversion of said nutrient source. An intracellular conversion is understood to comprise the synthesis of a biosynthetic product by fusion of said nutrient source - in the uptakable form such as a directly assimilable form or in a subsequently processed form - with a metabolite being synthesised by said cell, said fusion generating a metabolisable biosynthetic product. It is particularly preferred that the expressible enzyme activity according to the invention, when expressed, is mediating a biosynthetic reaction.

An altered expression of said first and/or second expressible enzyme activity in the microbial cell according to the invention shall be understood to comprise any expression that differs with respect to the rate of product formation or with respect to the amount of product formed as compared to a comparable microbial cell. Accordingly, if a wild-type microbial cell is subjected to the metabolic engineering manipulations according to the invention, the

skilled person will compare the expression of said first and/or second expressible enzyme activities provided in the metabolically engineered cell with the expression of the same activities in the wild-type microbial cell.

- 5 Generally, the person skilled in the art will preferably analyse - and compare with one another - similar or near identical microbial cells such as identical cells with and without an expressible enzyme activity according to the invention. This is standard laboratory practise and the person skilled in the art will know how to conduct such an analysis so that it may form a basis for a direct comparison of e.g. an expressed enzyme activity or an expressed  
10 coenzyme or an expressed redox system within the meaning of those terms as set out herein below.

- Preferably, the person skilled in the art will want to compare microbial cells to cells of at least the same species and more preferably to compare said cells to cells of at least the same  
15 subspecies.

- Accordingly, if an isolated microbial cell such as e.g. an industrial strain or a strain in a culture collection is subjected to the metabolic engineering manipulations according to the invention, the skilled person will compare the expression of said first and/or second expressible enzyme activities provided in the metabolically engineered cell with the expression of  
20 the same activities in the industrial strain or the microbial cell of the culture collection.

- The skilled artisan will know how to culture comparable strains such as strains of the same species or subspecies under identical or substantially similar conditions so as to provide a  
25 basis for performing the comparison between the relevant enzyme activities. The person skilled in the art will also know how to perform an enzymatic assay for use in said comparison and being indicative of the formation of a biosynthetic product resulting from the action of said first and/or second expressible activities, when expressed, and he will be aware of the potential of transcriptional and/or translational fusions in monitoring expression of said  
30 expressible enzyme activities under comparable conditions. The skilled person will also be able to perform immunoassays including quantitative immunoprecipitations. An analysis of gene expression is available in e.g. Old and Primrose (1985): Principles of Gene Manipula-



Accordingly, in a particularly preferred embodiment there is provided a microbial cell, preferably a yeast cell, wherein said first expressible enzyme activity is a metabolite synthase activity, more preferably an allosteric metabolite synthase activity, and even more preferably a glutamate synthase activity and wherein said second expressible enzyme activity is a metabolite synthetase activity, preferably a glutamine synthetase activity.

In another preferred embodiment is said first and/or second expressible enzyme activity a ligase activity or an NADH-dependent glutamate dehydrogenase activity or a NADPH-dependent glutamate dehydrogenase activity.

5 The microbial cell according to the invention may - in addition to an expressible first and/or second enzyme activity - also comprise a third expressible enzyme activity, said activity, when expressed in said microbial cell, preferably a yeast cell, is controlling assimilation in said cell of a nutrient source, said expression of said third enzyme activity in said microbial cell, preferably a yeast cell, being either novel or altered as compared to the expression of  
10 said third enzyme activity in a comparable wild-type microbial cell or a comparable isolated microbial cell, said third expressible enzyme activity being non-identical to any and both of said first and second expressible enzyme activities.

There is also provided an embodiment of the invention wherein said third expressible enzyme activity has been deleted from and no longer is present in said cell. It is particularly preferred to delete said third expressible enzyme activity when the microbial cell is a yeast cell, but the activity may also be deleted from any other eukaryotic microbial cell or from a prokaryotic microbial cell.

20 Reference is made to the above-mentioned comments and arguments concerning a definition of terms such as assimilation, nutrient source, altered expression and comparable microbial cell. The interpretations indicated herein above also apply with respect to said expressible third enzyme activity.

25 Accordingly, in one preferred embodiment according to the invention, the third expressible enzyme activity is preferably a metabolite dehydrogenase activity, and more preferably a glutamate dehydrogenase activity which is either present in said microbial cell and preferably in a reduced amount, more preferably in a substantially reduced amount, or eliminated from said cell by means of e.g. deletion of a nucleotide sequence encoding said activity or  
30 by effectively repressing the expression of said expressible third enzyme activity.

Consequently, an altered expression in said microbial cell according to the invention of said third expressible enzyme activity, preferably a glutamate dehydrogenase activity and even



more preferably a NADPH dependent glutamate dehydrogenase activity, shall be understood to comprise a decreased expression as compared to the expression of said activity in a comparable microbial cell. Accordingly, the expression of said third expressible enzyme activity, when expressed, is decreased by at least 1 percent, such as decreased by at least 2 percent, for example 4 percent, such as 6 percent, for example at least 8 percent, for example at least 10 percent, such as 12 percent, for example 14 percent, such as 16 percent, such as at least 18 percent, for example at least 20 percent, such as 22 percent, for example 24 percent, such as 26 percent, such as at least 28 percent, for example at least 30 percent, such as 32 percent, for example 34 percent, such as 36 percent, for example 38 percent, such as at least 40 percent, for example 42 percent, such as 44 percent, for example 46 percent, such as 48 percent, such as at least 50 percent, for example 52 percent, such as 54 percent, for example 56 percent, such as 58 percent, such as at least 60 percent, for example 62 percent, such as 64 percent, for example 66 percent, such as 68 percent, such as at least 70 percent, for example 72 percent, such as 74 percent, for example 76 percent, such as 78 percent, such as at least 80 percent, for example 82 percent, such as 84 percent, for example 86 percent, such as at least 88 percent, for example 90 percent, such as 92 percent, for example 94 percent, such as 96 percent, for example at least 98 percent, such as 99 percent, for example 99.2 percent, such as at least 99.4 percent, for example 99.6 percent, such as 99.8 percent, for example 99.9 percent, such as 99.92 percent, for example 99.94 percent, such as 99.96 percent, for example 99.98 percent, such as 99.99 percent, for example decreased to such an extent that said expression is unassayable using standard state of the art assays and/or said expression is effectively repressed and/or substantially eliminated.

However, an altered expression shall not be limited to a decreased expression. An increased expression of said third expressible enzyme activity shall also be understood to be comprised by the term altered expression.

In a particularly preferred embodiment of the invention said glutamate dehydrogenase activity is that of a *GDH* encoded polypeptide of *Saccharomyces cerevisiae* such as e.g. TN1, or an activity functionally equivalent therewith. A functionally equivalent activity is any activity capable of carrying out the same reaction with the provision of a similar outcome as that resulting from the reaction being carried out by the above-mentioned *GDH* encoded polypeptide of *Saccharomyces cerevisiae*. When the glutamate dehydrogenase activity is

A microbial cell according to the invention, such as a microbial cell, preferably a yeast cell, wherein the expression of said first and/or second expressible enzyme activity is increased, preferably substantially increased, whereas the expression of said third expressible enzyme activity is decreased, preferably substantially decreased, may in one embodiment produce a first metabolite, such as e.g. ethanol, said production of said first metabolite, preferably ethanol, is increased as compared to an expression of said metabolite in a comparable wild-type or isolated cell, said increase is increased by a factor of at least 1.005, for example 1.010, such as 1.015, for example 1.020, such as a factor of at least 1.025, for example a factor of at least 1.030, such as 1.035, for example 1.040, such as 1.045, for example 1.050, such as 1.055, for example 1.060, such as at least 1.065, for example a factor of at least 1.070, such as 1.075, for example 1.080, such as 1.085, for example a factor of at least

1.090, such as 1.095, for example 1.100, such as 1.105, for example 1.110, such as 1.115, for example 1.120, such as at least 1.125, for example a factor of at least 1.130, such as 1.135, for example 1.140, for example 1.145, such as 1.150, for example 1.155, such as at least 1.160, for example a factor of at least 1.165, such as a factor of at least 1.170, for example a factor of at least 1.175, such as 1.180, for example 1.185, such as 1.190, for example 1.195, such as a factor of at least 1.200, for example 1.21, such as 1.22, for example 1.23, such as 1.24, for example 1.25, such as 1.26, for example 1.27, such as 1.28, for example 1.29, such as a factor of at least 1.30, for example 1.35, such as 1.40, for example 1.45, such as 1.50, for example 1.55, such as 1.60, for example 1.65, such as 1.70, for example 1.75, such as 1.80, for example 1.85, such as 1.90, for example 1.95, such as a factor of at least 2.0, for example 2.2, such as 2.4, for example 2.6, such as 2.8, for example 3.0, such as 3.2, for example 3.4, such as 3.6, for example 3.8, such as at least 4.0, for example 4.2, such as 4.4, for example 4.6, such as 4.8, for example at least 5.0, such as 5.2, for example 5.4, such as 5.6, for example 5.8, such as at least 6.0, for example 6.2, such as 6.4, for example 6.6, such as 6.8, for example at least 7.0, for example 7.2, such as 7.4, for example 7.6, such as 7.8, for example 8.0, such as 8.2, for example 8.4, such as 8.6, for example 8.8, such as at least 9.0, for example 9.2, such as 9.4, for example 9.6, such as 9.8, for example a factor of at least 10.0.

Said microbial cell, most preferably yeast, having an increased production of a first metabolite has, in another preferred embodiment, a decreased production of a second metabolite, preferably glycerol. Said decreased production of said second metabolite, preferably glycerol, is decreased by at least 0.5 percent, for example at least 1 percent, such as at least 2 percent, for example 4 percent, such as 6 percent, such as at least 8 percent, for example at least 10 percent, such as 12 percent, for example 14 percent, such as 16 percent, such as at least 18 percent, for example at least 20 percent, such as 22 percent, for example 24 percent, such as 26 percent, such as at least 28 percent, for example at least 30 percent, such as 32 percent, for example 34 percent, such as 36 percent, for example 38 percent, such as at least 40 percent, for example 42 percent, such as 44 percent, for example 46 percent, such as 48 percent, such as at least 50 percent, for example 52 percent, such as 54 percent, for example 56 percent, such as 58 percent, such as at least 60 percent, for example 62 percent, such as 64 percent, for example 66 percent, such as 68 percent, such as at least 70 percent, for example 72 percent, such as 74 percent, for example 76 percent, such as 78 percent, such as at least

25 In a further embodiment, the microbial cell comprising said first and/or second expressible enzyme activity and optionally said third expressible enzyme activity, if said activity has not been eliminated from said cell by removal, deletion or otherwise, may further optionally comprise a fourth expressible enzyme activity. Accordingly, there is provided, in one embodiment of the invention, a microbial cell according to the invention further comprising said  
30 fourth expressible enzyme activity whereas, in another embodiment, said fourth expressible activity is not present in said microbial cell.

30 By exerting any one of the above-mentioned actions, said fourth expressible enzyme activity is controlling a redox system. The above-described maintenance of said redox system may well lead to an increased rate of synthesis of any one or more components of said system. Said maintenance may also lead to an increase in the pool of any one component being

comprised in said redox system, notably an increase in the pool of the reduced and/or oxidised form of a coenzyme.

Maintenance shall also be understood to comprise any effect that leads to a decrease in the pool of components of a redox system, notably a decrease in the pool of the reduced and/or oxidised form of a coenzyme.

The person skilled in the art will know how to assess an increase or decrease of any form of a coenzyme or of any redox system and he will know that he must compare the levels of that same coenzyme or redox system in a comparable wild-type microbial cell or an isolated microbial cell grown under identical or substantially similar conditions that allow for a direct comparison of said levels by exploiting state of the art monitoring techniques such as those described by Weuster and de-Graff (1996) in Adv. Biochem. Eng. Biotechnol., vol. 54, pages 75 – 108 and by Wiechert and de-Graff (1996) in Adv. Biochem. Eng. Biotechnol., vol. 54, pages 109 – 154. The person skilled in the art will preferably analyse similar or near identical microbial cells with and without said fourth expressible enzyme activity.

Accordingly, the terms increase and decrease relate to a level of expression or synthesis or to a concentration of a coenzyme and/or a redox system. The term level is used interchangeably in the art with terms such a synthesis rate and concentration. The person skilled in the art will be familiar with such terms and attach the correct meaning to their use in different contexts.

By acting to increase and/or decrease the rate of synthesis and/or the pool of a redox system component, the fourth expressible enzyme activity is controlling said intracellular redox system. The term alteration in this context shall be understood to comprise any change or deviation from the presence and/or amount of a redox system present in a comparable wild-type microorganism or an a comparable isolated microbial cell.

Any redox system can generally be perceived to contribute to the provision of a certain redox level in a cell. The totality of all such redox systems in a cell determines the redox level of said cell. The redox level of a cell thus comprises the presence and/or amount of the to-

talities of a reducing power and an oxidising power present in said cell. Accordingly, an alteration of an intracellular redox system can be measured either by monitoring the increase or decrease of a specific redox system, i.e. an increase or decrease in both the oxidised form as well as in the reduced form of a coenzyme constituting said redox system, or alternatively,  
5 said alteration can be monitored by measuring an overall cellular redox level.

In one preferred embodiment according to the invention, said fourth expressible enzyme activity provides the means for transferring reducing or oxidizing potential from one redox system to another. Alteration of an intracellular redox system may thus result in an increase  
10 or decrease in either the oxidised form or the reduced form of a coenzyme. It may also result in either one of those forms being increased while the other form is decreased. Any such alteration can be measured either by monitoring the increase or decrease of any one specific redox system, i.e. an increase or decrease in either an oxidised form and/or a reduced form of said coenzyme. Alternatively, said alteration can be monitored by measuring an overall  
15 intracellular redox level.

The person skilled in the art will be aware of such alterations leading to an increased or decreased redox level and, as exemplified herein and explained further in detail below, the skilled person will be aware of the state of the art techniques available for monitoring an  
20 intracellular redox level.

In one embodiment therefore, when said fourth expressible enzyme activity is present in said microbial cell and expressed therein, it results in an increased or decreased level, preferably an increased level, of at least one intracellular coenzyme in its oxidised or reduced form. Said coenzyme in its oxidised/reduced form is preferably selected from the group  
25 consisting of FAD/FADH<sub>2</sub>, NAD<sup>+</sup>/NADH and NADP<sup>+</sup>/NADPH.

Accordingly, the level of at least one intracellular coenzyme in its oxidised or reduced form is either increased or decreased, preferably increased, by a factor of at least 1.005, for  
30 example 1.010, such as 1.015, for example 1.020, such as a factor of at least 1.025, for example a factor of at least 1.030, such as 1.035, for example 1.040, such as 1.045, for example 1.050, such as 1.055, for example 1.060, such as at least 1.065, for example a factor of at least 1.070, such as 1.075, for example 1.080, such as 1.085, for example a factor of

- at least 1.090, such as 1.095, for example 1.100, such as 1.105, for example 1.110, such as 1.115, for example 1.120, such as at least 1.125, for example a factor of at least 1.130, such as 1.135, for example 1.140, for example 1.145, such as 1.150, for example 1.155, such as at least 1.160, for example a factor of at least 1.165, such as a factor of at least 1.170, for example a factor of at least 1.175, such as 1.180, for example 1.185, such as 1.190, for example 1.195, such as a factor of at least 1.200, for example 1.21, such as 1.22, for example 1.23, such as 1.24, for example 1.25, such as 1.26, for example 1.27, such as 1.28, for example 1.29, such as a factor of at least 1.30, for example 1.35, such as 1.40, for example 1.45, such as 1.50, for example 1.55, such as 1.60, for example 1.65, such as 1.70, for example 1.75, such as 1.80, for example 1.85, such as 1.90, for example 1.95, such as a factor of at least 2.0, for example 2.2, such as 2.4, for example 2.6, such as 2.8, for example 3.0, such as 3.2, for example 3.4, such as 3.6, for example 3.8, such as at least 4.0, for example 4.2, such as 4.4, for example 4.6, such as 4.8, for example at least 5.0, such as 5.2, for example 5.4, such as 5.6, for example 5.8, such as at least 6.0, for example 6.2, such as 6.4, for example 6.6, such as 6.8, for example at least 7.0, for example 7.2, such as 7.4, for example 7.6, such as 7.8, for example 8.0, such as 8.2, for example 8.4, such as 8.6, for example 8.8, such as at least 9.0, for example 9.2, such as 9.4, for example 9.6, such as 9.8, for example a factor of at least 10.0.
- Although an increase is preferred, it shall be understood that the term alteration is by no means limited to an increase in the level of at least one intracellular coenzyme in its oxidised or reduced form. Said alteration shall also comprise any decrease in the level of at least one intracellular coenzyme in its oxidised or reduced form.
- In an embodiment of the invention wherein the expression of said fourth expressible enzyme activity results in an increase or a decrease in the level i.e. concentration of an intracellular redox system as a whole, i.e. an increase or decrease of both of two corresponding oxidised and reduced forms of a coenzyme, said alteration is an increase or decrease, preferably an increase, by a factor of at least 1.005, for example 1.010, such as 1.015, for example 1.020, such as a factor of at least 1.025, for example a factor of at least 1.030, such as 1.035, for example 1.040, such as 1.045, for example 1.050, such as 1.055, for example 1.060, such as at least 1.065, for example a factor of at least 1.070, such as 1.075, for example 1.080, such as 1.085, for example a factor of at least 1.090, such as 1.095, for example 1.100, such as



The pyridine nucleotide transhydrogenase activity is either endogenous or heterologous to said microbial cell wherein it is expressed and said activity is either exhibited by a polypep-

tide which is membrane-bound in a natural host organism or located i.e. present in the cytoplasm of a natural host organism, said natural host organism preferably being selected from the group of mammalian cells, plant cells, eukaryotic and prokaryotic cells including microbial and bacterial cells such as e.g. Gram-positive microbial prokaryote and a Gram-negative microbial prokaryote.

Expression of said pyridine nucleotide transhydrogenase activity in said microbial cell in one preferred embodiment of the invention results in an increased conversion of NADPH and NAD to NADH and NADP. In another embodiment said expression of said pyridine nucleotide transhydrogenase activity results in an increased consumption of NADPH and an increased formation of NADH. In another embodiment the expression has the effect of increasing the consumption of NADH and increasing the formation of NADPH. In yet another embodiment the expression results in an increased formation of NADH and/or NADP. However, said expression may also result in a decreased formation of NADPH and/or a decreased NADPH/NADP ratio. The pyridine nucleotide transhydrogenase activity is preferably expressed in said microbial cell under anaerobic growth conditions and preferably results in the ratio of NADPH/NADP being lower than the ratio of NADH/NAD.

In another preferred embodiment, the transhydrogenase activity, preferably an activity that is membrane-bound in a natural host organism, is inserted into the plasma membrane of a microbial cell, preferably a yeast cell. The transhydrogenase activity would mediate a reaction consuming NADP and generating NADPH. In a particularly preferred embodiment, an *E. coli* transhydrogenase is expressed in a yeast cell and leads to an increased level of NADPH. The expression of the *E. coli* transhydrogenase is coupled to a proton gradient across the membrane of the natural host organism and a similar coupling is likely to be established when the membrane-bound *E. coli* transhydrogenase enzyme is integrated into the plasma membrane of a yeast cell such as e.g. *Saccharomyces cerevisiae*.

In a particularly preferred embodiment, the fourth expressible enzyme activity is that of CTH comprised in *Saccharomyces cerevisiae* strain TN4 deposited under Accession Number DSM 12267.

5 In yet another embodiment of the invention, the microbial cell has been metabolically engineered as described above and is capable of alternative NADH re-oxidation. Said alternative NADH re-oxidation is mediated at least by the combined expression of said above-mentioned first and second expressible enzyme activities. In one preferred embodiment said alternative NADH-reoxidation is mediated by overexpression of said first and second expressible enzyme activities in a microbial cell having a substantially decreased expression of said  
10 third expressible enzyme activity, or a microbial cell wherein said expression has been repressed or eliminated or deleted. Said fourth expressible enzyme activity may optionally be expressed concomitantly with an overexpression of said first and second expressible enzyme activities and a substantially reduced and preferably eliminated expression of said third expressible enzyme activity. In one preferred embodiment of the invention, said first, second,  
15 third and fourth expressible enzyme activities are those of a glutamate synthase, a glutamine synthetase, a glutamate dehydrogenase and a transhydrogenase, respectively.

Alternative NADH re-oxidation shall be understood to comprise the introduction of a novel major pathway for NADH re-oxidation or a generation of a substantially altered pathway for NADH-reoxidation in a microbial cell. Alteration in respect of a pathway for alternative NADH oxidation shall be understood in the context of the rate of a reaction mediating a conversion of one metabolite to another, said reaction also resulting in NADH re-oxidation. The rate of said re-oxidation reaction in a microbial cell capable of alternative NADH re-oxidation is substantially increased as compared to the rate of said reaction in a comparable microbial cell. The definition of the term comparable in respect of microbial cells is already introduced herein above.

Alternative NADH re-oxidation is an example of a microbial cell wherein the expression of a transhydrogenase, in combination with several additionally preferred expressible enzyme activities, are capable of generating a purposeful redesigning of a complex network of metabolic reactions. The redesigned microbial cell is invented by replacing or supplementing a normally dominant first metabolic reaction with a second reaction that is normally insignifi-

cant in relation to reaction rate and/or product formation as compared to said first dominant reaction. However, by significantly increasing said second reaction while at the same time significantly decreasing or even eliminating said first reaction, it is possible to achieve an alternative NADH re-oxidation. In a further embodiment of the invention, there is also provided a microbial cell capable of alternative NADPH re-oxidation or alternative NADP re-  
5 reduction.

In another aspect of the invention, there is provided a composition comprising the microbial cell and a carrier, preferably a physiologically acceptable carrier and more preferably a wa-  
10 ter-based liquid such as a broth suitable for culturing said microbial cell. The composition in a preferred embodiment is a fermentation starter culture.

There is also provided the aspect of a nucleotide sequence encoding a novel and industrially relevant transhydrogenase enzyme activity, said sequence comprising SEQ ID NO:1, as  
15 illustrated herein below, or part thereof, including functionally equivalent derivatives thereof encoding a transhydrogenase enzyme activity, preferably but not limited to conservative nucleotide substitutions and/or nucleotide deletions and/or nucleotide insertions. Said functionally equivalent derivatives may thus be at least 99.9 percent identical to SEQ ID NO:1, such as at least 99.8 percent identical to SEQ ID NO:1, for example at least 99.7 per-  
20 cent identical, such as at least 99.6 percent identical, for example at least 99.5 percent identical, such as at least 99.4 percent identical, for example at least 99.3 percent identical, such as at least 99.2 percent identical, for example at least 99.1 percent identical, such as at least 99 percent identical to SEQ ID NO:1, for example at least 98.5 percent identical to SEQ ID NO:1, such as at least 98.0 percent identical, for example 97.5 percent identical, such as at  
25 least 97.0 percent identical to SEQ ID NO:1, for example at least 96.5 percent identical, such as at least 96.0 percent identical, for example at least 95.5 percent identical, such as at least 95.0 percent identical, for example at least 94.5 percent identical, such as at least 94.0 percent identical, for example at least 93.5 percent identical, such as at least 93.0 percent identical to SEQ ID NO:1, for example at least 92.5 percent identical, such as at least 92.0  
30 percent identical, for example at least 91.5 percent identical, such as at least 91.0 percent identical, for example at least 90.5 percent identical, such as at least 90.0 percent identical, for example at least 85.0 percent identical to SEQ ID NO:1. In one embodiment, SEQ ID NO:1 is a sequence that is synthesised partly or wholly *in vitro*.

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comprising conservative amino acid substitutions.

Said production of said first metabolite is preferably substantially increased as compared to the production of said first metabolite in a comparable wild-type cell or a comparable isolated microbial cell. Accordingly, said microbial cell production of said first metabolite is increased at least by a factor of 1.02, such as a factor of at least 1.04, for example 1.06, such as 1.08, for example 1.10, such as at least 1.12, for example 1.14, such as 1.16, for example 1.18, such as 1.2, for example 1.25, such as 1.3, for example 1.4, such as 1.5, for example 1.6, such as 1.7, for example 1.8, such as 1.9, for example 2.0, such as 2.25, for example 2.5, such as 3, for example 3.5, such as a factor of at least 4, for example 4.5, such as 5, for example 6, such as 7, for example 8, such as 9, for example 10, such as 15, for example 20, such as 25, for example 30, such as 35, for example 40, such as 50, for example 60, such as 80, for example at least 100, such as 150, for example 200, such as 250, for example 300,

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In one embodiment, the microbial cell or the composition according to the invention is preferably used in a production of a first metabolite or used in a method of generating alternative intracellular NADH re-oxidation. Accordingly, the microbial cell is providing a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NADH re-oxidation for the purpose of providing, supplementing and/or increasing a pool of intracellular NAD, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

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In another embodiment there is provided a microbial cell for use in a production of a first metabolite, said cell harbouring a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NAD reduction for the purpose of providing, supplementing and/or increasing a pool of intracellular NADH, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

In yet another embodiment of the invention, there is provided a microbial cell for use in the production of a first metabolite, said cell harbouring a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NADPH re-oxidation for the purpose of providing, supplementing and/or increasing a pool of intracellular NADP, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

In a still further embodiment of the invention, there is provided a microbial cell for use in the production of a first metabolite, said cell harbouring a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NADP reduction for the purpose of providing, supplementing and/or increasing a pool of intracellular NADPH, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

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In a preferred embodiment there is provided a use of a microbial cell wherein said production of said first metabolite is substantially increased as compared to the production of said first metabolite in a comparable wild-type cell or a comparable isolated microbial cell. Said production of said first metabolite is increased at least by a factor of 1.01, such as 1.02, for example 1.03, such as a factor of at least 1.04, for example 1.05, such as 1.06, for example 1.07, such as 1.08, for example 1.09, such as 1.10, for example 1.11, such as at least 1.12, for example 1.14, such as 1.16, for example 1.18, such as 1.2, for example 1.25, such as 1.3, for example 1.4, such as 1.5, for example 1.6, such as 1.7, for example 1.8, such as 1.9, for example 2.0, such as 2.25, for example 2.5, such as 3, for example 3.5, such as a factor of at least 4, for example 4.5, such as 5, for example 6, such as 7, for example 8, such as 9, for example 10, such as 15, for example 20, such as 25, for example 30, such as 35, for example 40, such as 50, for example 60, such as 80, for example at least 100, such as 150, for example 200, such as 250, for example 300, such as 350, for example 400, such as 500, for example 600, such as 800, for example at least 1000, such as 1500, for example 2000, such

as 2500, for example 3000, such as 3500, for example 4000, such as at least 5000, for example 6000, such as 8000, for example at least 10000, such as 15000, for example 20000, such as at least 25000, for example 30000, such as 35000, for example 40000, such as a factor of at least 50000.

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It is preferred that the microbial cell is a yeast cell or a prokaryotic microbial cell and that said first metabolite is an alcohol or an acid, preferably ethanol, acetic acid, lactic acid or propionic acid.

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In a preferred use there is provided a microbial cell, preferably a yeast cell, further producing a second metabolite, said production of said second metabolite being substantially decreased as compared to the production of said second metabolite in a comparable wild-type cell or a comparable isolated microbial cell. Particularly preferred is a use wherein said second metabolite is glycerol or an undesirable aroma component naturally produced by a

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lactic acid bacterial cell.

The production of said second metabolite, preferably glycerol or an undesirable aroma component produced by a lactic acid bacterial cell, is reduced in a preferred use of said microbial cell by at least by at least 2 percent, such as 4 percent, for example at least 6 percent, such as 8 percent, for example at least 10 percent, such as 12 percent, for example 14 percent, such as 16 percent, for example 18 percent, such as at least 20 percent, for example 24 percent, such as at least 30 percent, for example 35 percent, such as at least 40 percent, for example 50 percent, such as 60 percent, for example at least 70 percent, such as 80 percent, for example at least 90 percent, such as decreased by at least 92 percent, for example 94 percent, such as 96 percent, for example 98 percent, such as decreased by 99 percent or decreased to such an extent that said second metabolite is virtually unassayable using state of the art assays for identifying and/or quantifying said second metabolite.

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Another preferred use of said microbial cell is in preparation of a drinkable or edible product or in a production of a first metabolite for use in said drinkable or edible product, said first metabolite having and/or providing a desirable organoleptic quality to said product. Preferably the first metabolite is ethanol or, when the microbial cell is a lactic acid bacterial

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A much preferred use of said microbial cell in said preparation of said drinkable or edible product is that of a microbial cell according to the invention, preferably a yeast cell or a lactic acid bacterial cell, further producing a second metabolite, said production of said second metabolite being substantially decreased as compared to the production of said second metabolite in a comparable wild-type cell or a comparable isolated microbial cell, said decreased production resulting in a provision of a desirable organoleptic quality to said product, said decrease is at least 2 percent, such as 4 percent, for example at least 6 percent, such as 8 percent, for example at least 10 percent, such as 12 percent, for example 14 percent, such as 16 percent, for example 18 percent, such as at least 20 percent, for example 24 percent, such as at least 30 percent, for example 35 percent, such as at least 40 percent, for example 50 percent, such as 60 percent, for example at least 70 percent, such as 80 percent, for example at least 90 percent, such as decreased by at least 92 percent, for example 94 percent, such as 96 percent, for example 98 percent, such as decreased by 99 percent or decreased to such an extent that said second metabolite is virtually unassayable using state of the art assays for identifying and/or quantifying said second metabolite.

20 There is also provided a use of a microbial cell, preferably a yeast cell or a lactic acid bacterial cell, in a preparation of a functional food.

In a yet further aspect of the invention there is provided a method of producing a first metabolite, said method comprising the steps of

i) cultivating a microbial cell in a suitable growth medium and under such conditions that said microbial cell is producing a first metabolite

and optionally

ii) isolating said first metabolite in a suitable form,

and further optionally

iii) purifying said isolated first metabolite.

The method comprises the culturing of any microbial cell including a microbial eukaryote and a microbial prokaryote. Among microbial eukaryotes, yeast cells and fungal cells are preferred, such as yeast cells like e.g. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* and the like, as well as algae such as e.g. *Chlamydomonas reinhardtii*, slime moulds such as e.g. *Dictyostelium discoideum* and filamentous fungi. Preferred filamentous fungi according to the method are species of *Neurospora* and *Aspergillus* such as e.g. *Neurospora crassa*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus cryzae* and *Penicillium chrysogenum*. Particularly preferred are many industrially relevant yeast cells, slime moulds and filamentous fungi providing a source of production of products such as e.g. antibiotics, steroids, pigments, enzymes, organic alcohols and acids, amino acids, polysaccharides and the like.

The method also pertains to the culturing of microbial prokaryotes such as Gram-positive species such as e.g. *Bacillus subtilis*, *Bacillus thuringensis*, *Bacillus licheniformis*, *Bacillus lentus* and *Bacillus stearothermophilus* and Gram-negative species such as *Escherichia coli*. Particularly preferred are also lactic acid bacterial species such as e.g. *Lactococcus lactis*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc* species, *Lactobacillus* species, *Pediococcus* species and similar industrially relevant species like e.g. *Bifidobacterium*.

Embodiments of this aspect of the invention comprise a method wherein said first metabolite is either a primary metabolite or a secondary metabolite. The metabolite may be produced in a cell also capable of further producing e.g. an endogenous or a heterologous product selected from the group consisting a protease, an amylase, a cellulase, a  $\beta$ -glucanase, an endoglucanase, a phosphatase, a xylanase, a lipase, a  $\beta$ -lactamase, a  $\beta$ -galactosidase, a  $\beta$ -glucuronidase, and a xylosidase. When the microbial cell is a lactic acid bacterium, said metabolite is preferably diacetyl, acetoin, or lactic acid.

The method according to the invention pertains in one embodiment to an increased production of said first metabolite, such as a substantially increased production, as compared to the



There is also provided a method for generating an alternative re-oxidation of a reduced co-enzyme, said method, in one embodiment, consisting essentially of providing in a microbial cell a novel or, in terms of efficiency and/or overall rate of reaction, a much improved pathway for alternative NADH and/or NADPH re-oxidation for use in providing, supplementing and/or increasing a pool of intracellular NAD and/or NADP, said provision, supplementation and/or increase being used in a process of altering, directing and/or redirecting the flux of primary and/or secondary metabolites in said cell.

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v) introducing said operably linked nucleotide sequence obtained under i), and optionally the nucleotide sequence obtained under ii), into said microbial cell obtained under iii) wherein said third expressible enzyme activity has been eliminated.

The expression signal may be further regulated by an upstream activating sequence (UAS), by an enhancer element or by a silencer element. The person skilled in the art will be aware of general molecular biology techniques for use in the construction in vitro of a recombinant DNA molecule. Such techniques are described e.g. in Sambrook et al. (1989) and in Old and Primrose (ibid.). Said skilled artisan will further be aware of the academic literature including general textbooks on molecular biology and genetic engineering and he will be able to combine various expression signals such as putative or recognised promoter regions with a range of regulatory nucleotide sequences generally known to exert an effect on the level of gene expression. The skilled person is able to monitor gene expression by construction of suitable transcriptional and/or translational fusions of an expression signal to a reporter gene generally available in the art. An expression signal can be a cloned expression signal or an in vitro synthesised expression signal. Expression signals in prokaryotic microbial cells are known to comprise so-called -35 and -10 regions and numerous examples of such regions are available from various databases.



30 In one embodiment of the method, the microbial cell is a yeast cell and the first expressible enzyme activity is a glutamate synthase activity, preferably a glutamate synthase activity encoded by *GLT1* of *Saccharomyces cerevisiae* TN17 as deposited under DSM Accession

Number 12275, or a functionally equivalent activity. The second expressible enzyme activity is a glutamine synthetase activity, preferably a glutamine synthetase activity encoded by *GLN1* of *Saccharomyces cerevisiae* TN15 as deposited under DSM Accession Number 12274, or a functionally equivalent activity. The third expressible enzyme activity, when present in said cell, is a glutamate dehydrogenase activity and preferably an activity encoded by *GDH1* of *Saccharomyces cerevisiae*. There is also provided a method wherein said cell is *Saccharomyces cerevisiae* TN19 as deposited under DSM Accession Number 12276. The method may comprise a further step of freezing or freeze-drying the microbial cell in the preparation of a reconstitutable lyophilisate.

In one presently preferred embodiment the microbial cell is a lactic acid bacteria as exemplified herein, and said fourth expressible enzyme activity is, at least in its native host organism, a cytoplasmic transhydrogenase, said expression of said cytoplasmic transhydrogenase resulting in an altered and/or novel product formation and/or metabolite production of said lactic acid bacteria.

An example of said novel product and/or metabolite formation is given herein below. Lactic acid bacteria metabolise pyruvate through a number of different pathways. The metabolite is converted into lactate by lactate dehydrogenase, into acetyl-CoA and CO<sub>2</sub> by pyruvate dehydrogenase, into formate by pyruvate formate lyase and into acetolactate and CO<sub>2</sub> by acetolactate decarboxylase. The carbon flux distribution through these pathways is dependent on the external growth conditions. This control is exerted through changes in the intracellular NADH/NAD<sup>+</sup> ratio (C. Garrigues, P. Loubiere, N. D. Lindley and M. Cacaïgn-bousquet (1997). *J. Bacteriology* **179**, 5282-5287; F. Lopez de Felipe, M. Kleerebezem, W. M. de Vos and J. Hugenholtz (1998). *J. Bacteriology* **180**, 3804-3808). This is illustrated by the observations from physiological studies of lactic acid bacteria that are listed below.

Lactic acid bacteria with an increased formation of the secondary metabolite diacetyl are relevant for a number of industrial applications. It has been shown that overexpression of NADH-oxidase from *Streptococcus mutans* in *L. lactis* results in a shift from homolactic (production of lactic acid) to mixed acid fermentation (production of lactic acid, acetic acid, acetoin and diacetyl) under aerobic growth conditions. This effect is ascribed to a decrease in the intracellular NADH/NAD<sup>+</sup> ratio of the recombinant strain (F. Lopez de Felipe, M.

The invention will be further exemplified in the below provided examples directed to preferred embodiments of the invention. It will be understood that the invention is by no means limited to said examples. The examples include figures illustrating the invention and the legends to said figures are listed below.

### FIGURE LEGENDS

Figure 1 shows plasmid pCHA1-GDH2 integration into the GDH2 locus.

5 Figure 2 shows plasmid pPGK-GDH2 integration into the GDH2 locus.

Figure 3 shows the specific enzyme activities of the NADPH-dependent and NADH-dependent glutamate dehydrogenases in protein extracts from biomass samples withdrawn from the continuous cultivations of strains TN1, TN9 and TN12 with increasing amounts of serine in the feed. Dotted line, ● : Gdh1p activity in TN1. Normal line, ● : Gdh2p activity in TN1. Normal line, ■ : Gdh2p activity in TN9. Normal line, Δ : Gdh2p activity in TN12.

Figure 4 shows the specific uptake rates of ammonium and serine in the continuous cultivation of strains TN1, TN9 and TN12 with increasing amounts of serine in the feed. Dotted lines: uptake of ammonium. Normal lines: uptake of serine. ● : TN1, ■ : TN9, Δ : TN12.

Figure 5 shows the measured specific production rates of glycerol (normal lines) and the calculated specific production rates of glycerol (dotted lines) in the continuous cultivations of strains TN1, TN9 and TN12 with increasing amounts of serine in the feed. ● : TN1, ■ : TN9, △ : TN12.

Figure 6 shows the specific uptake rates of glucose (dotted lines) and the specific production rates of ethanol (normal lines) in the continuous cultivations of strains TN1, TN9 and TN12 with increasing amounts of serine in the feed.

25 ● : TN1, ■ : TN9, Δ : TN12.

Figure 7 shows the consumption of glucose (■), and production of ethanol (●), glycerol (Δ), acetate (◇) and carbon dioxide (×) versus time in one of the anaerobic, glucose-limited batch cultivations of strain TN1 with ammonium as nitrogen source.

Figure 8 shows the consumption of glucose (■), and production of ethanol (●), glycerol (Δ), acetate (◇) and carbon dioxide (×) versus time in one of the anaerobic, glucose-limited batch cultivations of strain TN1 with serine as nitrogen source.

Figure 9 shows plasmids pPGK-GLN1 and pPGK-GLT1 constructed in this study and used to integrate the strong constitutive promoter of PGK into the chromosome in front of GLN1 and GLT1, respectively.

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Figure 10 shows the natural logarithm of the biomass concentrations of strains TN1(O), TN9 ( $\Delta$ ), TN17 ( $\blacksquare$ ) and TN19 ( $\blacklozenge$ ) versus time during exponential growth in the anaerobic, glucose-limited batch cultivations. The equations show the slopes and intersections with the second axis of the trendlines through the measured values. The slopes are equal to the maximum specific growth rates of the strains.

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Figure 11 shows the consumption of glucose ( $\blacktriangle$ ) and production of ethanol ( $\square$ ), glycerol ( $\blacklozenge$ ) and carbon dioxide ( $\times$ ) in one of the anaerobic batch cultivations of strain TN1.

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Figure 12 shows the consumption of glucose ( $\blacktriangle$ ) and production of ethanol ( $\square$ ), glycerol ( $\blacklozenge$ ) and carbon dioxide ( $\times$ ) in one of the anaerobic batch cultivations of strain TN9.

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Figure 13 shows the consumption of glucose ( $\blacktriangle$ ) and production of ethanol ( $\square$ ), glycerol ( $\blacklozenge$ ) and carbon dioxide ( $\times$ ) in one of the anaerobic batch cultivations of strain TN19.

## EXAMPLE 1

### Expression of NADH-dependent and NADPH-dependent glutamate dehydrogenase activities in yeast.

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#### Introduction

This example comprises a study of strains of *Saccharomyces cerevisiae* with a deletion in *GDH1* and a concomitant constitutive or inducible overexpression of *GDH2*. The effect on growth rates, enzyme activities and product formation is reported. Batch and continuous cultivations of the novel genetically engineered strains were carried out in high performance bioreactors.

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## Materials and methods

**Microorganisms and their maintenance.** All *Saccharomyces cerevisiae* strains were generated from *Saccharomyces cerevisiae* T23D. The strain was kindly provided by Jack Pronk from the Department of Microbiology and Enzymology, Kluyver Laboratory of Biotechnology, Delft University of Technology, The Netherlands. The yeast strains were maintained at 4°C on YPG agar plates, monthly prepared from a lyophilised stock kept at -80°C.

*Escherichia coli* DH5α (F<sup>-</sup> F80dlacZ DM15 D(lacZYA- argF) U169 deoR recA1 endA1 hsdR17(r<sub>k</sub><sup>-</sup> m<sub>k</sub><sup>+</sup>) supE44 l<sup>-</sup> thi-1 gyra96 relA1) (GIBCO BRL, Gaithersburg, MD, USA) was used for subcloning.

**Preparation of DNA.** Plasmid DNA from *E. coli* was prepared with Qiagen columns (Qiagen GmbH, Düsseldorf, Germany) following the manufacturer's instructions. For the purification of DNA fragments used for cloning experiments, the desired fragments were separated on 0.8% agarose gels, excised and recovered from agarose using the Qiagen DNA isolation kit (Qiagen GmbH, Düsseldorf, Germany). Chromosomal DNA from *Saccharomyces cerevisiae* was extracted as follows. Cells were grown in medium in shake flasks and harvested at OD=1.5. 10 mg of wet cells were resuspended in 0.5 ml Tris-Cl (pH 8.0) and quenched with 0.5 ml glass beads (size 250-500 microns) in the presence of 0.5 ml Tris-saturated phenol (pH 8.0). The DNA was extracted from the phenol phase with chloroform, precipitated with 98% ethanol and resuspended in TE buffer. RNA in the extract was removed by treatment with RNAaseA (purchased from Promega) and finally the DNA was purified by precipitation with ethanol/lithium chloride and resuspended in TE buffer. The DNA primers were purchased from DNA Technology (Aarhus, Denmark).

**Deletion of *GDH1*.** Plasmid pGDH1del was kindly donated by professor F.K. Zimmermann (Boles *et al.*, 1993). In pGDH1del a 1.0 kb fragment of *GDH1* has been replaced by a 1.1 kb fragment containing the open reading frame of *URA3*. The construct was linearised with ClaI/PvuII prior to transformation. Correct deletion of *GDH1* was verified by PCR analysis and by measurements of GDH1p activity in protein extracts from transformants. No NADPH-dependent glutamate dehydrogenase activity could be detected in correct transformants.

**Overexpression of *GDH2*.** The *CHA1* promoter was cloned by PCR using pfu polymerase (New England Biolabs) and the primers CHA1start (5'-ATT CAT CGA TGA ATT CTA TCT TAT GGT CCC ATT CTT TAC TGC ACT GTT TAC A-3'), SEQ ID NO:3, consisting of restriction enzyme sites for ClaI and EcoRI in front of nucleotides -364 to --329 upstream of *CHA1*) and CHA1stop (5'-GGC CAC TAG TGA TAT CAA AGC ATT CTC TCG CTG GTT AAT TTT CCT GTC TCT TGT CTA TCA GCA CTT AAA AA-3'), SEQ ID NO:4, consisting of restriction enzyme sites for SpeI, EcoRV and BsmI in front of nucleotides -1 to -45 upstream of *CHA1*). The resulting DNA fragment was isolated after gel electrophoresis on a 0.8% agarose gel and subcloned into the SmaI site in vector pUC19, resulting in plasmid pCHA1. The *CHA1* promoter was isolated by from pCHA1 by digestion with BsmI and HincII (located in the multi-cloning site of pUC19). Plasmid YEpMSP3, containing the open reading frame of *GDH2* (Boles *et al.*, 1993), was kindly donated by Professor F.K. Zimmermann. The plasmid was digested with MscI and BsmI and ligated with the HincII/BsmI *CHA1p* fragment, resulting in a plasmid with insertion of the *CHA1* promoter in front of the *GDH2* start codon. This construct was digested with BamHI and a fragment, consisting of the *CHA1* promoter and 2.27 kb of the open reading frame of *GDH2*, was isolated. Plasmid pFA6A-kanMX3 contains the gentamicin resistance gene, *G418<sup>r</sup>*, flanked by two direct repeats and two multi-cloning sites (Wach *et al.*, 1994). A EcoRI/MscI DNA fragment, consisting of nucleotides -500 to -136 upstream of *GDH2*, was isolated from YEpMSP3 and inserted into pFA6A-kanMX3, digested with EcoRI and EcoRV. The resulting construct was linearised by digestion with BamHI and ligated with the 2.64 kb *CHA1p-GDH2* fragment, resulting in plasmid pCHA1GDH2 (Figure 1). pCHA1GDH2 was linearised with SpeI/AatII prior to transformation. It was verified by PCR that the *CHA1* promoter was inserted in front of the open reading frame of *GDH2* on chromosome IV of correct transformants. For this purpose primers CHA1start and GDH2verif (5'-GGT TTT CTA CAA TCT CCA AAA GAG-3'), SEQ ID NO:5, spanning the region from nucleotides 1294 to 1271 of the *GDH2* open reading frame.

Primers Gdh2start (5'-GCG CGA GAT CTT CTA GAA TGC TTT TTG ATA ACA AAA AT-3'), SEQ ID NO:6, containing restriction enzyme sites for BglII and XbaI in front of nucleotides 1 to 21 of *GDH2*, and Gdh2stop (5'-CGC GCA GAT CTC CGC GGA GAG CCT AAA CGA TTA ACA AA-3'), SEQ ID NO:7, containing restriction enzyme sites for

BglII and SacII in front of nucleotides 1221 to 1201 of *GDH2*, were used to clone parts of the structural gene of *GDH2* by PCR with pfu polymerase (New England Biolabs). A DNA fragment of the correct size was isolated from a 0.8% agarose gel after electrophoresis and digested with BglII overnight. The fragment was ligated into the unique BglII digestion site of plasmid Yep24-pPGK behind the *PGK* promoter and in front of the *PGK* terminator (Walfridsson *et al.*, 1997), resulting in plasmid Yep24-pPGK-GDH2. A 2.65 kb SmaI/SacII DNA fragment, consisting of the *PGK* promoter and the cloned part of *GDH2* was isolated from Yep24-pPGK-GLT1. The fragment was ligated into plasmid pFA6A-kanMX3 (Wach *et al.*, 1994), digested with EcoRV and SacII, resulting in plasmid pPGK-GDH2 (Figure 2). The plasmid was linearised by digestion with TthIII1 prior to transformation. Correct insertion of the plasmid into the *GDH2* locus on chromosome IV was verified by PCR analysis of chromosomal DNA extracted from transformants with resistance towards genitcin. For this purpose primers PGKverif (5'- GTC ACA CAA CAA GGT CCT A-3'), SEQ ID NO:8, spanning the region from nucleotides -420 to -400 upstream of the *PGK* start codon, and Gdh2verif (described above) were used.

**Transformation of *E. coli* and *S. cerevisiae*.** *E. coli* DH5 $\alpha$  was transformed by electrotransformation using the Bio-Rad electroporation equipment (Biorad Laboratories, Richmond, USA). Transformants were selected on L broth plates containing 100 mg/ml ampicillin. *S. cerevisiae* cells were made competent for plasmid uptake by treatment with lithium acetate and polyethyleneglycol (Schiestl & Gietz., 1989). 5  $\mu$ g of DNA was used for each transformation. Transformants were plated directly on selective media except for the G418 resistant transformants. These were suspended in YPD for 24 hours prior to plating on selective media in order to obtain expression of the G418 resistance gene. Correct integration of the fragments from pH0del and pSUC2 into the chromosome was verified by PCR analysis using extracted DNA from the transformants.

**Medium in the batch and continuous cultivations.** The strains of *S. cerevisiae* were cultivated in a mineral medium prepared according to Verduyn *et al.* (1990). Vitamins were added by sterile filtration following heat sterilisation of the medium. The concentrations of glucose and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> initially in the batch cultivations were 25 g per l and 3.75 g per l, respectively. The concentration of glucose in the feed to the continuous cultivations was 25 g per l while the concentration of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was varied from 3.75 g per l to 0 g per l. The



**Experimental set-up for the batch and continuous cultivations.** Anaerobic batch and continuous cultivations were performed at 30°C and at a stirring speed of 800 rpm in in-house manufactured bioreactors. The working volume of the batch reactors and the continuous cultivation reactors were 4.5 litres and 1.0 litres, respectively. pH was kept constant at 5.00 by addition of 2 M KOH. The bioreactors were equipped with off-gas condensers cooled to 2°C. The bioreactors were continuously sparged with N<sub>2</sub> containing less than 5 ppm O<sub>2</sub>, obtained by passing N<sub>2</sub> of a technical quality (AGA 3.8), containing less than 100 ppm O<sub>2</sub>, through a column (250x30 mm) filled with copper flakes and heated to 400°C. The column was regenerated daily by sparging it with H<sub>2</sub> (AGA 3.6). A mass flow controller (Bronkhorst HiTec F201C) was used to keep the gas flow into the bioreactors constant at 0.50 l nitrogen per min per litre Norprene tubing (Cole-Parmer Instruments) was used throughout in order to minimise diffusion of oxygen into the bioreactors. The bioreactors were inoculated to an initial biomass concentration of 1 mg per l with precultures grown in un baffled shake flasks at 30°C and 100 rpm for 24 hours. The anaerobic batch cultivations of strains TN1, TN9, TN12 and TN22 were each carried out three times with identical results. Steady state in the continuous cultivations was obtained after growth for 10-11 residence times. This was verified by measuring a constant formation of CO<sub>2</sub> and medium components, e.g. ethanol, glycerol and acetate, by the yeast throughout 2-3 residence times.

**Determination of dry weight.** Dry weight was determined gravimetrically using nitrocellulose filters (pore size 0.45  $\mu\text{m}$ ; Gelman Sciences). The filters were predried in a microwave oven (Moulinex FM B 935Q) for 10 min. A known volume of culture liquid was filtered and the filter was washed with an equal volume of demineralised water followed by drying in a microwave oven for 15 min. The relative standard deviation (RSD) of the determinations was less than 1.5 % based on triple determinations ( $n=3$ ).

**Analysis of medium compounds.** Cell-free samples were withdrawn directly from the bioreactor through a capillary connected to a 0.45  $\mu\text{m}$  filter. Samples were subsequently stored at  $-40^{\circ}\text{C}$ . Glucose, ethanol, glycerol, acetic acid, pyruvic acid, succinic acid and 2-oxoglutarate were determined by HPLC using an HPX-87H Aminex ion exclusion column (RSD < 0.6 %,  $n=3$ ). The column was eluted at  $60^{\circ}\text{C}$  with 5 mM  $\text{H}_2\text{SO}_4$  at a flow rate of 0.6 ml per min. Pyruvic acid, acetic acid and 2-oxoglutarate were determined with a Waters 486 UV meter at 210 nm whereas the other compounds were determined with a Waters 410 refractive index detector. The two detectors were connected in series with the UV detector first. The  $\text{CO}_2$  concentration in the off-gas was determined using a Brüel & Kjær 1308 acoustic gas analyser (RSD = 0.02 %) (Christensen *et al.*, 1995). Ammonium was determined using a commercially available assay (Boehringer Mannheim Cat.No. 1 112 732). Serine was determined as described by Barkholt and Jensen, 1989. In a separate experiment the off gas from the bioreactor was bubbled through liquid nitrogen and the ethanol concentration in the frozen mixture of water, ethanol and acetaldehyde was determined by HPLC after evaporation of the  $\text{N}_2$ . Hereby the loss of ethanol through the reflux condenser of the bioreactor was determined to be between 4% and 9% of the ethanol formed by the bioreaction depending on the dilution rate (Schulze, 1995). In the carbon balances the measured ethanol fluxes were corrected for this loss through evaporation.

**Measurement of enzyme activities.** Culture liquid was withdrawn from the bioreactor into an ice cooled beaker, centrifuged and washed twice with 10 mM potassium phosphate buffer (pH 7.5,  $2^{\circ}\text{C}$ ) containing 2 mM EDTA. Subsequently the cells were resuspended in 4.2 ml 100 mM potassium phosphate buffer (pH 7.5,  $2^{\circ}\text{C}$ ) containing 2 mM  $\text{MgCl}_2$  followed by immediate freezing in liquid nitrogen and storage at  $-40^{\circ}\text{C}$ . Prior to analysis 0.22 ml of 20

mM DTT was added to the samples whereafter they were distributed into precooled 2 ml eppendorf tubes containing 0.75 ml glass beads (size 0.25-0.50). The cells were disrupted in a bead mill for 12.5 min. (0°C). The test tubes were centrifuged (20000 rpm, 20 min., 0°C) whereafter the supernatants were pooled in one test tube. During the following analyses the extract was kept on ice. Enzyme assays were performed at 30°C using a Shimadzu UV-260 spectrophotometer at 30°C. Reaction rates, corrected for endogenous rates, were proportional to the amount of extract added. All enzyme activities are expressed as micromole of substrate converted per minute per mg total cellular protein as determined by the Lowry method. Glutamate dehydrogenase (NAD<sup>+</sup> and NADP<sup>+</sup>) (EC 1.4.1.5 and EC 1.4.1.4, respectively) were assayed as described by Bruinenberg *et al.* (1983a). Glutamate synthase (GOGAT) (EC 1.4.1.14) was assayed as described by Holmes *et al.* (1989).

## Results

**Construction of strains with a deletion in *GDH1* and an overexpression of *GDH2*.** The object of the study was to analyse whether the NADH-dependent glutamate dehydrogenase, encoded by *GDH2*, could substitute the NADPH-dependent isoenzyme, encoded by *GDH1*, in assimilation of ammonium and 2-oxoglutarate into glutamate in *S. cerevisiae*. This should lead to a reduction in surplus formation of NADH in biomass synthesis and thus, to a *S. cerevisiae* strain with a reduced formation of glycerol and possibly an increased formation of ethanol. To obtain this, strain were constructed with a deletion in *GDH1* and an overexpression of *GDH2*. *GDH1* was deleted as described earlier (Boles *et al.*, 1993) in the haploid strain TN2 derived from *S. cerevisiae* CBS8066 (Nissen *et al.*, 1998). The resulting strain was denounced TN9. In order to obtain a strain with a stable, constitutive overexpression of *GDH2*, the promoter of *PGK*, encoding phosphoglycerate kinase, was inserted in front of the start codon of *GDH2* on chromosome IV. *PGK* encodes one of the most abundant mRNA and protein species in the cell, accounting for 1% to 5% of the total cellular mRNA and protein during growth on fermentative carbon sources (Dobson *et al.*, 1982). Insertion of the promoter was obtained by homologue recombination of a 4.8 kbp *SpeI*/xxx fragment from pPGKGDH2 into the *GDH2* locus in strain TN9 (see materials and methods). The resulting strain was denounced TN22. The promoter of *CHAI*, encoding the catabolic L-serine dehydratase, has been reported to be inducible by low amounts of serine (Bornæs *et al.*, 1993). To study to effect of varying levels of Gdh2p activity in a strain background with no

activity of Gdh1p, the *CHA1* promoter was inserted into chromosome IV in front of the open reading frame of *GDH2* in strain TN9. The serine inducible promoter was chosen to avoid the use of inducible promoters that were dependent on addition of a second carbon source besides glucose since this would complicate the comparison between cultivations with increasing concentrations of this second carbon source. Furthermore, the *CHA1* promoter was reported to be induced up to 130 times by addition of 5 mM serine, which would have very little influence on the cell physiology. The insertion of the *CHA1* promoter was obtained by homologue recombination of a 4.3 kbp *SpeI/AatII* fragment from pCHA1GDH2 into the *GDH2* locus in strain TN9 (see materials and methods). The resulting strain was denounced TN12.

**Continuous cultivations.** Physiological studies of the genetically engineered strains were carried out in anaerobic continuous cultivations. Strain TN12 with the inducible *CHA1* promoter inserted into the chromosome in front of *GDH2* was cultivated until steady states had been achieved in growth media containing glucose as the primary carbon source and increasing amounts of serine from 0 mM to 30 mM. The amount of ammonium sulphate in the feed was regulated to give a final concentration of 60 mM ammonium and serine. This was done to study the degree of *GDH2* induction that could be achieved with the new promoter and the effect of this induction on product formation. Similar cultivations of strains TN1 and TN9 were performed. Hereby, the effects of the increasing amounts of serine in the feed, the deletion of *GDH1*, and the insertion of the new promoter in front of *GDH2* on the product formation could be discriminated from each other.

The specific activities of Gdh1p, Gdh2p and Glt1p were measured *in vitro* in cell extracts from each steady-state cultivation. The activity of Gdh1p in TN1 decreased almost linear from 0.657 to 0.475 units per mg total cellular protein (U per mg TCP) when the serine content in the feed was increased from 0 mM to 30 mM (dotted line in Figure 3). This fitted well with the measured decrease in ammonium uptake from 18.8 to 8.2 mmol per c-mole biomass per hour, respectively. Earlier studies of *S.cerevisiae* have shown that a decrease in ammonium uptake results in a simultaneous reduction in Gdh1p activity in cell free extracts (ter Schure *et al.*, 1995a). No activity of the NADPH-dependent glutamate dehydrogenase could be detected in cell free extracts from any of the continuous cultivations of TN9 and

TN12. The Gdh2p activity in cell free extracts of strain TN1 increased from 0.007 U per mg TCP when ammonium was used as the sole nitrogen source to 0.022 U per mg TCP when 30 mM serine was present in the feed (Figure 3). Deletion of *GDH1* in TN9 resulted in a marked increase in the specific activity of Gdh2p. When ammonium was used as the sole nitrogen source the activity was determined to 0.268 U per mg TCP, an increase of a factor 40 as compared to the activity in TN1. It has been shown that the intracellular level of glutamine results in strong repression of *GDH2* expression at the transcriptional level (Miller and Magasanik, 1991). When *GDH1* is deleted the synthesis of glutamate from 2-oxoglutarate and ammonium is strongly affected which probably leads to a significant decrease in the intracellular concentration of the component. Since glutamate is one of the substrates in glutamine synthesis this will result in a further decrease in the intracellular level of the amino acid. Thus, the strong increase in Gdh2p in TN9 probably was due to an elevation of glutamine repression of *GDH2*. Addition of increasing amounts of serine to the feed resulted in a significant reduction in the Gdh2p activity. Since the residual glucose concentrations in all cultivations of TN9 was constant at 20 c-mmoles per litre this was not due to glucose repression of *GDH2* (Coschigano *et al.*, 1991). The residual concentration of ammonium decreased from 48 mM when the compound was used as the sole nitrogen source to 28 mM when 30 mM serine was added to the medium. In an earlier study in continuous cultivations of a wild-type *S. cerevisiae* it was demonstrated that the Gdh2p activity decreased by a factor 4 when the residual ammonium concentration was reduced by this amount (ter Schure *et al.*, 1995b). Since the intracellular glutamine concentration decreased simultaneously, this effect was not due to repression from this component. Instead, an observed increase in the intracellular concentration of 2-oxoglutarate might explain the reduction in Gdh2p activity since this component generally is thought to be the product of the reaction catalysed by the enzyme when the cells are cultivated on nitrogen sources other than ammonium. No increase in the specific activity of Gdh2p was observed in the cultivation of TN12 with ammonium as the sole nitrogen source. This clearly demonstrated that the increase of Gdh2p activity observed under these growth conditions in TN9 was due to deregulation of the *GDH2* promoter. The expected induction of the *CHA1* promoter by serine was not observed in the cultivations of TN12 when increasing amounts of the component was added to the feed. The presence of 5 mM serine in the medium increased the Gdh2p activity by a factor of 6 while the maximum induction was observed by addition of 30 mM serine. Here the activity of the enzyme was 13 times higher than when ammonium was used as nitrogen



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**Table 2**

Ammonium in the feed, mM	60	55	45	30
Serine in the feed, mM	0	5	15	30
Ethanol	0.525	0.532	0.550	0.572
Glycerol	0.062	0.044	0.028	0.012
Pyruvate	0.004	0.003	0.002	0.003
Acetate	0.000	0.000	0.000	0.000
Succinate	0.003	0.004	0.003	0.004
Carbon dioxide	0.257	0.267	0.274	0.274
Biomass	0.123	0.122	0.124	0.106
Total	0.974	0.972	0.981	0.971

**Table 2.** Product yields in the continuous cultivations of strain TN9 with increasing amounts of serine in the feed. Unit: c-moles product per c-moles of glucose and serine consumed.

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**Table 3**

Strain	TN12				TN22
Ammonium in the feed, mM	60	55	45	30	60
Serine in the feed, mM	0	5	15	30	0
Ethanol	0.500	0.529	0.534	0.546	0.516
Glycerol	0.061	0.045	0.026	0.014	0.030
Pyruvate	0.002	0.002	0.001	0.001	0.002
Acetate	0.004	0.004	0.004	0.004	0.004
Succinate	0.003	0.003	0.003	0.004	0.004
Carbon dioxide	0.247	0.279	0.250	0.265	0.255
Biomass	0.125	0.128	0.130	0.138	0.138
Total	0.942	0.990	0.948	0.972	0.949

**Table 3.** Product yields in the continuous cultivations of strain TN12 with increasing amounts of serine in the feed and in the continuous cultivation of strain TN22 with ammonium as the sole nitrogen source. Unit in the cultivations of TN12: c-moles product per c-moles of glucose and serine consumed. Unit in the cultivation of TN22: c-moles product per c-mole glucose consumed.

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Carbon balances of the products yields in the 12 steady state cultivations showed that 95% - 98% of the consumed carbon was converted into one of the listed products. The degree of reduction of the remaining 2% - 5% of the consumed carbon varied between 0 and 1, indicating that the production of carbon dioxide was measured to low.

Ammonium and serine were consumed simultaneously by the three strains (Figure 3). Thus, the presence of both nitrogen sources in the growth medium did not result in repression of the specific uptake systems of the two components. The increasing amounts of serine added to the feed resulted in an increase in the specific uptake of the compound. A model based on simple Michaelis Menten type kinetics, describing the dependence of the uptake on the extracellular serine concentration, did not fit the measurements (results not shown). Thus, other components, *e.g.* glucose, probably had a regulatory effect on the specific uptake of the compound. The need for nitrogen uptake in the form of ammonium decreased when increasing amounts of serine were consumed by the cells. This resulted in a reduction in the specific ammonium uptake in the cultivations (Figure 4). The total specific uptake of nitrogen was calculated from the measurements of the ammonium and serine uptake to be constant at 17-18 mmoles nitrogen per c-mole biomass per hour in all 12 steady state continuous cultivations. This indicated a constant composition of the biomass with respect to the content of protein, RNA, DNA and carbohydrates

Addition of serine to the medium resulted in a decrease in the specific glycerol production (Figure 5). The serine addition reduces the cells need for *de novo* synthesis of the amino acid. Synthesis of one mole serine from glucose and ammonium involves the reduction of two moles  $\text{NAD}^+$  to NADH and a deamination step where one mole glutamate is converted to 2-oxoglutarate. As described earlier glutamate can be regenerated from 2-oxoglutarate by either two isoenzymes of glutamate dehydrogenase, encoded by *GDH1* and *GDH2*, under consumption of NADPH or NADH, respectively. Furthermore, glutamate can be synthesised from 2-oxoglutarate and glutamine by glutamate synthase, encoded by *GLT1*, under consumption of NADH (Cogoni *et al.*, 1995). In strain TN1 all three enzymes are present while only Gdh2p and Glt1p are active in strains TN9 and TN12. Thus, addition of serine results in a reduction of surplus NADH formation of either 1 or 2 moles per mole serine in TN1 and 1 mole per mole serine in TN9 and TN12. *De novo* synthesis of serine in *S. cerevi-*



*siae* CBS8066 was calculated to 157 c-mmoles per c-mol biomass based on measurements of the content of serine and the amino acids synthesised from serine in the protein pool (Schulze, 1995). By assuming this to be similar in TN1, TN9 and TN12 the specific serine synthesis during growth on ammonium as the sole nitrogen source was 19.9 c-mmoles per c-mole biomass per hour. In the cultivations with 15 mM and 30 mM serine in the feed the specific serine uptake exceeded this value (Figure 4). Thus, in these cultivations serine was also catabolised by the cells. As mentioned above L-serine dehydratase, encoded by *CHA1*, catalyses deamination of serine to pyruvate and ammonium. No increase in the secretion of pyruvate, acetate or metabolites in the tricarboxylic cycle was measured when increasing amounts of serine was added to the feed. Hence, pyruvate stemming from the deamination reaction was converted into acetaldehyde and further into ethanol under consumption of one mole NADH per mole serine. These two effects of serine addition to the medium on the reduction of surplus NADH formation resulted in the observed decrease in the specific glycerol production. This was quantified by extracting the reduction in surplus NADH formation due to serine addition from the value of the specific glycerol production measured in the continuous cultivations with ammonium as the sole nitrogen source (dotted lines in Figure 5). In all calculations a value for the reduction in surplus NADH formation of 1 mole per mole of serine was used. The reduction in the specific glycerol formation of strains TN9 and TN12 when cultivated in media with increasing amounts of serine in the feed could be accounted for by the two effects of serine addition on surplus NADH formation since the calculated values were close to equal with the measured values. For strain TN1 the calculated specific glycerol production was equal to the measured values in the cultivations with 15 mM and 30 mM serine in the feed. In the cultivation with 5 mM serine in the feed a correct value for the specific glycerol formation could only be calculated by assuming a reduction in surplus NADH formation of 2 moles per mole serine. Hereby a specific glycerol production of 61.2 c-mmoles per c-mole biomass per hour was obtained as compared to 68.6 c-mmoles per c-mole biomass per hour when a value for the reduction in surplus NADH formation of 1 mole per mole of serine was used. This indicates that Gdh1p catalyses the regeneration of glutamate from 2-oxoglutarate when the serine uptake is low while Gdh2p catalyses the reaction with increasing levels of the amino acid. Earlier studies support this observation since it has been shown that Gdh1p catalyses the formation of glutamate during growth on ammonium as the sole nitrogen source while Gdh2p catalyses the interconversion of glutamate and 2-oxoglutarate during growth on other nitrogen sources (Courchesne and

Magasanik, 1988; Miller and Magasanik, 1990 ). The calculations clearly demonstrated the significant effect of serine addition on the cell physiology but also that formation of surplus NADH in the synthesis of biomass has a high control on glycerol formation. Furthermore, they demonstrated that the decrease in the specific glycerol production was independent of the specific activity of Gdh2p.

The specific ethanol production was also affected by addition of serine to the feed (Figure 6). In the cultivations of TN1 the specific ethanol formation reached a maximum when 5 mM and 15 mM serine was added to the feed and then decreased slightly when the serine content was increased to 30 mM. In the cultivations of TN9 the specific ethanol production increased almost linearly from 540 to 626 c-mmoles per c-mole biomass per hour while it was constant in the cultivations of TN12. Serine addition has at least four potential influences on ethanol formation in *S. cerevisiae*. The cellular consumption of ATP is to a given extent affected by the increase in active uptake of serine, by the decrease in active uptake of ammonium and by the reduction in glycerol formation since glycerol synthesis involves consumption of one ATP per molecule. Since ATP is formed through synthesis of ethanol during anaerobic growth these effects will change the specific ethanol formation. Furthermore, as described above serine was catabolised to ethanol in some of the cultivations. The effects of serine addition on ethanol synthesis were quantified by assuming ATP consumption in the active uptake systems of serine and ammonium of one ATP per molecule. The calculated changes in ethanol formation due to the four effects was then used to calculate values for the specific ethanol formation with increasing amounts of serine in the feed from the value measured when ammonium was used as the sole nitrogen source. An increase in the specific ethanol production from 540 to 544 c-mmoles per c-mole biomass per hour and from 531 to 536 c-moles per c-mole biomass per hour was calculated for strains TN9 and TN12, respectively. For TN1 the calculated specific ethanol production decreased from 441 to 435 c-mmoles per c-mole biomass per hour. Thus, the listed direct effects of serine addition on ATP consumption and ethanol synthesis could not explain the changes in the specific ethanol production. Instead, the changes could be due to a switch in the metabolism leading to an increased ATP consumption in biomass synthesis. It has been shown in an earlier study that only glutamate synthase catalyses the formation of glutamate when *GDH1* is deleted (Miller and Magasanik, 1990). Thus, a strain with a double deletion in *GDH1* and *GLT1* grew poorly on ammonium as the sole nitrogen source. In the study a growth medium

containing high levels of glucose was used and hence, Gdh2p activity in the double mutant was repressed. By overexpressing *GDH2* in the mutant a specific activity of the gene product of 0.195 units per mg TCP was obtained which led to an increase in the growth rate on ammonium to wild-type levels. In the continuous cultivation of strain TN9, carrying the deletion in *GDH1*, with ammonium as the sole nitrogen source the specific Gdh2p activity was 0.268 units per mg TCP due to absence of glucose repression. This strongly indicated that glutamate was synthesised by both glutamate synthase and the NADH-dependent glutamate dehydrogenase under these growth conditions. When the serine content in the medium was increased the specific Gdh2p activity decreased. Since the specific growth rate was kept constant at  $0.127 \text{ h}^{-1}$  it is reasonable to assume that also the cellular glutamate synthesis rate was constant with increasing amounts of serine in the feed. Thus, increasing amounts of the component probably was synthesised through the reaction catalysed by glutamate synthase. Here 2-oxoglutarate and glutamine is converted into two molecules of glutamate under consumption of NADH. Glutamine is then regenerated from glutamate under consumption of ammonium and ATP catalysed by glutamine synthetase. In the net reaction catalysed by the two enzymes 2-oxoglutarate and ammonium are converted into glutamate under consumption of NADH and ATP which results in an increase in ATP consumption in biomass synthesis compared to glutamate synthesis catalysed by Gdh2p. Overall, the increase in the specific ethanol formation in strain TN9 with increasing amounts of serine in the feed might be due to this increase in ATP consumption in the synthesis of glutamate. In the cultivation of TN12 with ammonium as the sole nitrogen source glutamate synthase catalysed the synthesis of glutamate since the specific Gdh2p activity was measured to 0.005 units per mg TCP. As described earlier the specific activity of the NADH-dependent glutamate dehydrogenase increased with increasing amounts of serine in the feed but the level did not exceed 0.065 units per mg TCP which probably was too low to substitute glutamate synthase in formation of glutamate. Hence, ATP consumption in biomass synthesis remained constant in the cultivations, which resulted in a constant specific ethanol formation. The NADPH-dependent glutamate dehydrogenase catalysed glutamate formation in the cultivations of TN1. Thus, an increase in ATP consumption in the assimilation of ammonium could not explain the small increase in the specific ethanol formation observed in the cultivations with 5 mM and 15 mM serine in the feed. Instead, the observed increase of approximately 6% compared to the cultivation with ammonium as the sole nitrogen source could be due to the sum of small changes in the metabolism, resulting in an increased ATP con-

sumption in biomass synthesis. In the cultivation of TN9 with ammonium as the sole nitrogen source an increase in the specific ethanol production and a decrease in the specific glycerol production of 22% and 20%, respectively, was observed compared to the cultivation of TN1. Similar results were obtained in the cultivation of TN12. Thus, deletion of *GDH1*,  
5 resulting in formation of cellular glutamate by Glt1p, led to a *S. cerevisiae* strain with improved characteristics for ethanol production.

To further analyse the effect of *GDH2* overexpression in a *Δgdh1* mutant without the influence of serine addition a glucose-limited continuous cultivation of strain TN22 was carried out with ammonium as the sole nitrogen source at a dilution rate of  $0.11 \text{ h}^{-1}$ . As mentioned above TN22 has the strong, constitutive promoter of *PGK* inserted in front of *GDH2* on chromosome IV. A specific activity of 0.381 units per mg TCP of the NADH-dependent glutamate dehydrogenase was measured in cell-free extracts from the steady state cultivation of TN22 while the activities of Gdh1p and Glt1p was absent and 0.012 units per mg TCP, respectively. The carbon balance and the balance over the degree of reduction of the substrates and products measured in the cultivation both closed within 3.8 %. Thus, it was concluded that all products secreted by the cell had been quantified correctly. The high Gdh2p activity was reflected in the measured specific production rates of ethanol and glycerol. The two rates were measured to 411 c-mmoles per c-mole biomass per hour and 24 c-mmoles per c-mole biomass per hour, respectively. The low value for the specific ethanol production was similar to the value measured in the continuous cultivation of TN1 with ammonium as the sole nitrogen source. Hence, the level of ATP consumption in the assimilation of ammonium into glutamate probably was identical in the two strains. This indicated that in TN22 the NADH-dependent glutamate dehydrogenase completely had substituted the role of glutamate synthase in glutamate synthesis that was observed in strains TN9 and TN12. The very low specific glycerol production in TN22 compared to strain TN9 and TN12 was surprising. The specific uptake of ammonium in the cultivation was 19.2 mmoles per c-mole biomass per hour which was 12% higher than in TN9. Also the biomass yield increased from 123 c-mmoles per c-mole glucose in TN9 to 138 c-mmoles per c-mole glucose in TN22. This indicated that the high Gdh2p activity resulted in a higher flux from 2-oxoglutarate and ammonium to glutamate in TN22 than in TN9, which led to an increase in NADH reoxidation through the reaction catalysed by the enzyme, and thus, a decrease in formation of glycerol. Furthermore, it indicated that the reduction in biomass yields observ-

ed in the continuous cultivations of TN9 and TN12 compared to TN1 and TN22 (Tables 1-3) was due to a limitation in the ammonium assimilation. No changes in formation of the organic acids were observed compared to TN9 and TN12.

**Batch cultivations.** The anaerobic physiology of the genetically engineered *S. cerevisiae* strains were also studied in batch cultivations with glucose as the primary carbon source and either ammonium or serine as nitrogen source. This was done to quantify the effect of the genetic changes on the maximum specific growth rate,  $\mu_{\max}$ , and on the product yields.

10 The product yields obtained in the anaerobic batch cultivations of the four strains are listed  
in Table 4.

TABLE 4

Strain	TN1		TN9		TN12		TN22
Serine (mM)	0	60	0	60	0	60	0
Ammonium (mM)	60	0	60	0	60	0	60
Ethanol	0.480	0.491	0.520	0.534	0.526	0.531	0.500
Glycerol	0.097	0.067	0.060	0.037	0.066	0.040	0.067
Pyruvate	0.003	0.002	0.003	0.003	0.002	0.002	0.004
Acetate	0.003	0.028	0.004	0.018	0.019	0.022	0.001
Succinate	0.003	0.003	0.002	0.004	0.003	0.004	0.003
Carbon di-oxide	0.261	0.262	0.275	0.278	0.272	0.275	0.262
Biomass	0.121	0.119	0.114	0.105	0.072	0.107	0.126
Total	0.968	0.972	0.978	0.979	0.960	0.981	0.963
$\mu_{\max}$ (h <sup>-1</sup> )	0.41	0.31	0.22	0.20	0.17	0.24	0.39

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**Table 4.** Product yields in the anaerobic, glucose-limited batch cultivations of strains TN1, TN9, TN12 and TN12 with either ammonium or serine as nitrogen source. Unit in the cultivation with ammonium as nitrogen source: c-moles product per c-mole glucose consumed. Unit in the cultivation with serine as nitrogen source: c-moles product per c-moles glucose and serine consumed.

The glycerol yield decreased significantly when strain TN1 was cultivated with serine as nitrogen source as compared to the cultivation on ammonium. As for the continuous cultivations this was due to a reduction in surplus formation of NADH in biomass synthesis under these growth conditions. A dramatic increase in formation of acetate was observed and also the ethanol yield increased. The total cellular uptake of serine was 309 c-mmoles per c-mole biomass. By assuming the constant need for *de novo* serine synthesis of 157 c-mmoles per c-mole biomass used in the earlier calculations it could be calculated that 152 c-mmoles serine per c-mole biomass was catabolised to ethanol and acetate. The specific ethanol and acetate formation increased with 92 c-mmoles per c-mole biomass and 210 c-mmoles per c-mole biomass, respectively, when serine was used as nitrogen source instead of ammonium. Thus, degradation of serine to acetate could not account for the increase in the yield of the component alone. Formation of acetate by the NAD(P)<sup>+</sup>-dependent aldehyde dehydrogenase, encoded by *ALD2*, has been proposed to have a physiological role in reduction of NADP<sup>+</sup> to NADPH, which is consumed in biomass synthesis (Bruinenberg *et al.*, 1983b; Miralles and Serano, 1995). Serine is a precursor in synthesis of cysteine and phospholipids, which are synthesis pathways that require large amounts of NADPH. The presence of high concentrations of intracellular serine could potentially increase the flux through these pathways and account for the increase in NADPH formation through synthesis of acetate. The maximum specific growth rate of TN1 decreased significantly from 0.41 h<sup>-1</sup> to 0.31 h<sup>-1</sup> when the nitrogen source was switched from ammonium to serine. When serine is used as nitrogen source the nitrogen has to be made available for biomass synthesis through degradation of the amino acid to pyruvate and ammonium. This process is less efficient than direct uptake of ammonium followed by assimilation into glutamate catalysed by glutamate dehydrogenase and glutamate synthase and hence, the reduction in the specific growth rate probably was due to this difference in nitrogen assimilation. The production of ethanol, glycerol, acetate and carbon dioxide as functions of time is shown in Figures 7 and 8 for the cultivations of TN1 with ammonium and serine as nitrogen sources, respectively. In the cultivation on ammonium the carbon dioxide content in the exhaust gas decreased rapidly to zero within 40 minutes after depletion of glucose in the medium also formation of other products ended. Thus, the metabolism stopped when the available carbon source was consumed. In the cultivation with serine as nitrogen source the drop of the carbon dioxide content in the exhaust gas lasted 400 minutes after depletion of glucose. The measurements

strongly indicated that this was due to degradation of serine to pyruvate and further into acetate whereby carbon dioxide was formed since the concentration of acetate continued to increase after depletion of glucose while the concentrations of the other products remained constant (Figure 8). Due to capacity problems only the total consumption of serine in the cultivation was measured by quantifying the serine content in the start and end samples. Hence, it could not be calculated whether the acetate formation after glucose depletion was due to serine degradation. The slow decrease in carbon dioxide formation after depletion of glucose was not observed in the cultivations of TN9 and TN12 when serine was used as nitrogen source. This could indicate that acetate was produced in the cultivation of TN1 to form NADPH, which in turn was consumed in assimilation of ammonium, stemming from degradation of serine, by the NADPH-dependent glutamate dehydrogenase.

Biomass samples were withdrawn from the batch cultivations when the cells had reached the exponential growth phase and the specific activities of Gdh1p, Gdh2p and Glt1p were measured in the pool of proteins extracted from these samples (Table 5).

TABLE 5

Strain	TN1		TN9		TN12		TN22
Serine (mM)	0	60	0	60	0	60	0
Ammonium (mM)	60	0	60	0	60	0	60
Gdh1p	1.522	1.197	0	0	0	0	0
Gdh2p	0.020	0.221	0.055	0.218	0.006	0.329	0.625
Glt1p	0.030	0.048	0.045	0.045	0.050	0.040	0.040

Table 5. Specific enzyme activities of the NADPH-dependent and NADH-dependent glutamate dehydrogenases and glutamate synthase in protein extracts from biomass samples withdrawn in the exponential growth phase of strains TN1, TN9, TN12 and TN22 in the glucose-limited batch cultivations with either ammonium or serine as nitrogen source.

The specific activity of the NADPH-dependent glutamate dehydrogenase decreased from 1.522 to 1.197 units per mg TCP while that of the NADH-dependent isoenzyme increased

from 0.020 to 0.221 units per mg TCP when serine was used as nitrogen source instead of ammonium. Also a small increase in the specific activity of glutamate synthase was observed. This could indicate that a small shift occurred in the cofactor specificity of ammonium assimilation into glutamate towards consumption of both NADPH and NADH. Since the  
 5 reduction in the glycerol yield was accounted for by the effect on surplus NADH formation of serine consumption the major part of the intracellular ammonium probably still was assimilated under consumption of NADPH by Gdh1p.

Deletion of *GDH1* in TN9 resulted in a decrease in the specific growth rate to  $0.22 \text{ h}^{-1}$  when  
 10 ammonium was used as nitrogen source. The specific activities of Gdh2p and Glt1p in the exponential growth phase were 0.055 and 0.045 units per mg TCP, respectively, while no activity of Gdh1p could be detected. Thus, the reduction in the specific growth rate of the strain probably was due to a reduction in the rate of glutamate synthesis from ammonium and 2-oxoglutarate since the total specific activity of the two enzymes was more than ten  
 15 times lower than that of Gdh1p in TN1. The low activity of Gdh2p compared to the activity of the enzyme in the continuous cultivations was due to transcriptional repression by glucose (Coschigano *et al.*, 1991). As observed in the continuous cultivations assimilation of ammonium by a combination of glutamate synthase and the NADH-dependent glutamate dehydrogenase resulted in an increase in the ethanol yield and a decrease in the glycerol yield  
 20 due to consumption of NADH and ATP in the synthesis of glutamate as compared to consumption of only NADPH in strain TN1. When serine was used as nitrogen source the same qualitative changes in the yields of ethanol, glycerol and acetate was observed as in the cultivations of TN1. The increase in the acetate yield from 0.004 c-moles per c-mole glucose to 0.018 c-moles per c-mole glucose and serine was lower than observed in TN1, indicating  
 25 that part of the NADPH consumed by Gdh1p in TN1 was synthesised through oxidation of acetaldehyde to acetate by the cytoplasmic  $\text{NAD(P)}^+$ -dependent aldehyde dehydrogenase. The decrease in the biomass yield of TN9 was lower when serine was used as nitrogen source instead of ammonium. This was not observed in the cultivations of TN1 and TN12 and could not be explained by any of the measured changes in product yields or enzyme  
 30 activities.

The activities of Gdh2p and Glt1p in the exponential growth phase of TN12 were 0.006 and 0.050 units per mg TCP, respectively, when ammonium was used as nitrogen source. Thus,



the total activity of the enzymes in the ammonium assimilation was even lower than observed in TN9 and resulted in a further reduction in the maximum specific growth rate to  $0.15 \text{ h}^{-1}$ , almost a decrease to one third of  $\mu_{\text{max}}$  in TN1. Since activity of Gdh2p was close to zero in TN12 under these growth conditions the synthesis of glutamate was catalysed by glutamate synthase alone. This led to a small increase in the ethanol yield. As opposed to the observations from the cultivations of TN1 and TN9 on ammonium as nitrogen source a dramatic increase in the acetate yield and a corresponding reduction in the biomass yield was observed in TN12. The drop in the amount of biomass synthesised per mole of glucose suggested that a significant change in the anabolism of the cell had occurred. An important role of glutamate in the metabolism is donation of ammonium in synthesis of many amino acids through deamination to 2-oxoglutarate catalysed by the two-glutamate dehydrogenases. As described the specific activities of these were very low in TN12, which could limit the flux through the synthesis pathways to these amino acids and thus, the total flux towards biomass synthesis. The increase in acetate formation could be due to an increase in NADPH consumption in biomass synthesis. When serine was used as nitrogen source the specific activity of Gdh2p increased to 0.329 units per mg TCP. This represented an induction of the enzyme by a factor of more than 50 while the Gdh2p activity only increased ten times in TN1 when serine was added. Hence, the induction of the *CHA1* promoter by serine was functional in TN12. The activity of Glt1p decreased slightly to 0.040 units per mg TCP. The increased activity of the NADH-dependent glutamate dehydrogenase resulted in a marked increase in the maximum specific growth rate of the strain. This clearly illustrated that the growth rate of the strain was limited by the flux towards glutamate when ammonium was used as nitrogen source. Furthermore, it demonstrated that Gdh2p was able to substitute both Gdh1p and Glt1p in synthesis of glutamate from ammonium and 2-oxoglutarate. The high Gdh2p activity also resulted in a biomass yield comparable to the values obtained in the cultivations of TN1 and TN9. This supported the theory that the low biomass yield on ammonium was due to a limitation in biomass synthesis by the deamination reaction from glutamate to 2-oxoglutarate.

In order to analyse the effect of *GDH2* overexpression in TN9 without the influence of serine addition anaerobic batch cultivations of strain TN22 was carried out with glucose as carbon source and ammonium as nitrogen source. When the original *GDH2* promoter was substituted by the strong, constitutive *PGK* promoter in TN22, a Gdh2p activity in the ex-

ponential growth phase of 0.625 was obtained. This resulted in a dramatic increase in the maximum specific growth rate from  $0.22 \text{ h}^{-1}$  to  $0.39 \text{ h}^{-1}$  which was very close to  $\mu_{\max}$  in strain TN1. Again this illustrated that the NADH-dependent glutamate dehydrogenase could fully substitute the NADPH-dependent isoenzyme if expressed at a sufficient level.

- 5 The biomass yield in TN22 increased by 11% compared to TN9 and was even higher than in TN1. This resulted in a higher glycerol yield since the amount surplus NADH formed increased per c-mole of glucose that was consumed. The increase in the ethanol yield that was observed in TN9 was absent in the cultivation of TN22, which illustrated that glutamate synthesis from ammonium and 2-oxoglutarate, occurred without consumption of ATP in  
10 TN22.

- Concluding remarks.** The work described herein above was carried out to analyse if the activity of the *GDH1* encoded NADPH-dependent glutamate dehydrogenase could advantageously be substituted by the *GDH2* encoded NADH-dependent isoenzyme in a pro-  
15 cess involving assimilation of ammonia and 2-oxoglutarate into glutamate. Another ob-jective was to analyse the effect of such metabolically engineered ammonia assimilation on the product formation in anaerobic cultivations.

- The anaerobic batch cultivation of strain TN22 clearly demonstrated that high expression of  
20 *GDH2* in a *Δgdh1* mutant resulted in a strain wherein the physiological role of Gdh1p in a wild-type strain had been taken over by Gdh2p. Furthermore, it was shown that this change in the metabolism resulted in a reduction of glycerol formation. Thus, formation of surplus NADH in biomass synthesis exerts a major effect on glycerol synthesis. Most likely, a fur-ther reduction in the formation of undesirable metabolic products can be obtained by meta-  
25 bologically engineering other reactions catalysed by isoenzymes with different cofactor speci-ficities. The reduced glycerol yield did not result in an increase of the metabolic flux to-wards ethanol. Accordingly, in the metabolically engineered strains, the formation of etha-nol is not limited by the flux of metabolites towards other fermentation products.

- 30 An attempt was made to study the effect of *GDH2* overexpression on the anaerobic physio-logy in more detail by introducing the inducible promoter of *CHA1* in front of *GDH2* on chromosome IV. The induction of the promoter by serine was clearly evident, but very high amounts of the amino acid had to be added to the medium in order to obtain a high expres-

In this example, a new strategy is presented for optimisation of the ethanol yield in *Saccharomyces cerevisiae* through metabolic engineering. It is based on the physiological roles of glycerol and ethanol in oxidation of surplus NADH and in formation of ATP, respectively,

**Overexpression of *GLTI*.** Primers Glt1start (5'-GCG CGG GAT CCT CTA GAA TGC CAG TGT TGA AAT CAG AC-3'), SEQ ID NO:9, containing restriction enzyme sites for BamHI and XbaI in front of nucleotides 1 to 21 of *GLTI*, and Glt1stop (5'-CGC GCG GAT CCC CGC GGG CTG GAC CAT CCC AAG GTT CC-3'), SEQ ID NO:10, containing restriction enzyme sites for BamHI and SacII in front of nucleotides 1149 to 1169 of *GLTI*,

**Overexpression of *GLN1*.** Primers Gln1start (5'- GCG CGG GAT CCT CTA GAA TGG CTG AAG CAA GCA TCG AA-3'), SEQ ID NO:11, containing restriction enzyme sites for BamHI and XbaI in front of nucleotides 1 to 21 of *GLN1*, and Gln1stop (5'-CGC GCG GAT CCC CGC GGT TAT GAA GAT TCT CTT TCA AA-3'), SEQ ID NO:12, containing restriction enzyme sites for BamHI and SacII in front of nucleotides 1093 to 1113 of *GLN1*, were used to clone *GLN1* by PCR with the pfu polymerase (New England Biolabs). The obtained DNA fragment was used to construct plasmid pPGK-GLN1, containing *GLN1* behind the promoter of *PGK* inserted into pFA6A-kanMX3 (Figure 9), as described above for plasmid pPGK-GLT1. pPGK-GLN1 was linearised by digestion with KpnI prior to transformation. Correct insertion of the plasmid into the *GLN1* locus on chromosome XVI was verified by PCR analysis of chromosomal DNA extracted from transformants with resi-

**Overexpression of *GLN1*.** Primers Gln1start (5'- GCG CGG GAT CCT CTA GAA TGG CTG AAG CAA GCA TCG AA-3'), SEQ ID NO:11, containing restriction enzyme sites for BamHI and XbaI in front of nucleotides 1 to 21 of *GLN1*, and Gln1stop (5'-CGC GCG GAT CCC CGC GGT TAT GAA GAT TCT CTT TCA AA-3'), SEQ ID NO:12, containing restriction enzyme sites for BamHI and SacII in front of nucleotides 1093 to 1113 of *GLN1*, were used to clone *GLN1* by PCR with the pfu polymerase (New England Biolabs). The obtained DNA fragment was used to construct plasmid pPGK-GLN1, containing *GLN1* behind the promoter of *PGK* inserted into pFA6A-kanMX3 (Figure 9), as described above for plasmid pPGK-GLT1. pPGK-GLN1 was linearised by digestion with KpnI prior to transformation. Correct insertion of the plasmid into the *GLN1* locus on chromosome XVI was verified by PCR analysis of chromosomal DNA extracted from transformants with resi-

stance towards genitacin. For this purpose primers PGKverif, spanning the region 420 to 400 bp upstream of the *PGK* start codon, and GLN1verif, spanning the region from nucleotides 52 to 70 downstream of *GLT1*, were constructed.

- 5    **Transformation of *E. coli* and *S. cerevisiae*.** *E. coli* DH5 $\alpha$  was transformed by electrotransformation using the Bio-Rad electroporation equipment (Biorad Laboratories, Richmond, USA). Transformants were selected on L broth plates containing 100 mg/ml ampicillin. *S. cerevisiae* cells were made competent for plasmid uptake by treatment with lithium acetate and polyethyleneglycol (Schiestl & Gietz., 1989). 3  $\mu$ g of DNA was used for each  
10 transformation. Transformants were suspended in YPD for 24 hours prior to plating on YPD, containing 150 mg genitacin per litre, in order to obtain expression of the G418 resistance gene.

- 15    **Medium in the batch cultivations.** The strains of *S. cerevisiae* were cultivated in a mineral medium prepared according to Verduyn *et al.*(1990). Vitamins were added by sterile filtration following heat sterilisation of the medium. The concentrations of glucose and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> initially in the batch cultivations were 25 g per l and 3.75 g per l, respectively. Growth of *S. cerevisiae* under anaerobic conditions requires the supplementary addition to the medium of ergosterol and unsaturated fatty acids, typically in the form of Tween 80  
20 (Andreassen & Stier, 1953; Libudzisz *et al.*, 1986). Ergosterol and Tween 80 were dissolved in 96 % (v/v) ethanol and the solution was autoclaved at 121°C for 5 min. The final concentrations of ergosterol and Tween 80 in the medium were 4.2 mg per g DW and 175 mg per g DW, respectively. To prevent foaming 75  $\mu$ l per l antifoam (Sigma A-5551) was added to the medium.

- 25    **Experimental set-up for the batch cultivations.** Anaerobic batch cultivations were performed at 30°C and at a stirring speed of 600 rpm in in-house manufactured bioreactors. The working volume of the batch reactors was 4.5 litres. pH was kept constant at 5.00 by addition of 2 M KOH. The bioreactors were continuously sparged with N<sub>2</sub> containing less  
30 than 5 ppm O<sub>2</sub>, obtained by passing N<sub>2</sub> of a technical quality (AGA 3.8), containing less than 100 ppm O<sub>2</sub>, through a column (250x30 mm) filled with copper flakes and heated to 400°C. The column was regenerated daily by sparging it with H<sub>2</sub> (AGA 3.6). A mass flow controller (Bronkhorst HiTec F201C) was used to keep the gas flow into the bioreactors

constant at 0.50 l nitrogen min<sup>-1</sup> liter<sup>-1</sup> Norprene tubing (Cole-Parmer Instruments) was used throughout in order to minimise diffusion of oxygen into the bioreactors. The bioreactors were inoculated to an initial biomass concentration of 1 mg l<sup>-1</sup> with precultures grown in un baffled shake flasks at 30°C and 100 rpm for 24 hours. Ethanol evaporation from the bioreactors was minimised by off-gas condensers cooled to 2°C. The anaerobic batch cultivations of strains TN1, TN9, TN15, TN17 and TN19 were each carried out three times with identical results.

**Determination of dry weight.** Dry weight was determined gravimetrically using nitrocellulose filters (pore size 0.45 µm; Gelman Sciences). The filters were predried in a microwave oven (Moulinex FM B 935Q) for 10 min. A known volume of culture liquid was filtered and the filter was washed with an equal volume of demineralised water followed by drying in a microwave oven for 15 min. The relative standard deviation (RSD) of the determinations was less than 1.5 % based on triple determinations (n=3).

**Analysis of medium compounds.** Cell-free samples were withdrawn directly from the bioreactor through a capillary connected to a 0.45 µm filter. Samples were subsequently stored at -40°C. Glucose, ethanol, glycerol, acetic acid, pyruvic acid, succinic acid and 2-oxoglutarate were determined by HPLC using an HPX-87H Aminex ion exclusion column (RSD < 0.6 %, n=3). The column was eluted at 60°C with 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 ml min<sup>-1</sup>. Pyruvic acid, acetic acid and 2-oxoglutarate were determined with a Waters 486 UV meter at 210 nm whereas the other compounds were determined with a Waters 410 refractive index detector. The two detectors were connected in series with the UV detector first. The CO<sub>2</sub> concentration in the off-gas was determined using a Brüel & Kjær 1308 acoustic gas analyser (RSD = 0.02 %) (Christensen *et al.*, 1995). In a separate experiment the off gas from the bioreactor was bubbled through liquid nitrogen and the ethanol concentration in the frozen mixture of water, ethanol and acetaldehyde was determined by HPLC after evaporation of the N<sub>2</sub>. Hereby the loss of ethanol through the reflux condenser of the bioreactor was determined to be between 4% and 9% of the ethanol formed by the bioreaction depending on the dilution rate (Schulze, 1995). In the carbon balances the measured ethanol fluxes were corrected for this loss through evaporation.

**Measurement of enzyme activities.** Culture liquid was withdrawn from the bioreactor into an ice cooled beaker, centrifuged and washed twice with 10 mM potassium phosphate buffer (pH 7.5, 2°C) containing 2 mM EDTA. Subsequently the cells were resuspended in 4.2 ml 100 mM potassium phosphate buffer (pH 7.5, 2°C) containing 2 mM MgCl<sub>2</sub> followed by immediate freezing in liquid nitrogen and storage at -40°C. Prior to analysis 0.22 ml of 20 mM DTT was added to the samples whereafter they were distributed into precooled 2 ml eppendorf tubes containing 0.75 ml glass beads (size 0.25-0.50). The cells were disrupted in a bead mill for 12.5 min. (0°C). The test tubes were centrifuged (20000 rpm, 20 min., 0°C) whereafter the supernatants were pooled in one test tube. During the following analyses the extract was kept on ice. Enzyme assays were performed at 30°C using a Shimadzu UV-260 spectrophotometer at 30°C. Reaction rates, corrected for endogenous rates, were proportional to the amount of extract added. All enzyme activities are expressed as micromole of substrate converted per minute per mg total cellular protein as determined by the Lowry method. Glutamate dehydrogenase (NAD<sup>+</sup> and NADP<sup>+</sup>) (EC 1.4.1.5 and EC 1.4.1.4, respectively) were assayed as described by Bruinenberg *et al.* (1983a). Glutamine synthetase (EC 6.3.1.2) and glutamate synthase (GOGAT) (EC 1.4.1.14) was assayed as described by Holmes *et al.* (1989).

## Results

**Metabolic control analysis.** Anaerobic physiology in continuous cultivations of *S. cerevisiae* CBS8066 has previously been analysed by means of metabolic flux analysis and said theoretical although practically applicable analysis showed that the flux through the reaction catalysed by the NADPH-dependent glutamate dehydrogenase (reaction 25 in a proposed model) was 8.0 c-mmoles per g biomass per hour at a dilution rate of 0.3 h<sup>-1</sup>, although the net flux from 2-oxoglutarate to glutamate was only 2.0 c-mmoles per g biomass per hour.

Accordingly, if Glt1p and Gln1p catalysed the same reaction instead of Gdh1p, one mole of NADH and one mole of ATP would be expected to be consumed per mole of glutamate synthesised, instead of consumption of one mole NADPH. The result of such a metabolic engineering would consequently be a reduction in a surplus formation of NADH.



A decrease in the formation of glycerol from 9.8 to 5.0 c-mmoles per g biomass per hour, a reduction by 49%, was predicted. Furthermore, the consumption of ATP was hypothesised to result in a 6% increase of ethanol formation, from 54.9 to 58.2 c-mmoles per g biomass per hour.

5

**Construction of new strains.** Earlier the haploid *S. cerevisiae* strain TN1 was constructed from a diploid progeny of the industrial model strain *S. cerevisiae* CBS8066 (Nissen *et al.*, 1998b). This was done by deletion of *HO*, encoding an endonuclease involved in mating type switching, and isolation of stable haploids following sporulation of deletion mutants.

- 10 Furthermore, *GDH1* was deleted in TN1 as described earlier, resulting in strain TN9 (Nissen *et al.*, 1998a). In this study the object was to construct new strains from TN9 with a stable, constitutive overexpression of *GLN1*, encoding glutamine synthetase, and *GLT1*, encoding glutamate synthase. This was achieved by inserting the strong, constitutive promoter of *PGK* in front of the two genes on chromosomes XVI and IV, respectively. *PGK* encodes
- 15 one of the most abundant mRNA and protein species in the cell, accounting for 1% to 5% of the total cellular mRNA and protein during growth on fermentative carbon sources (Dobson *et al.*, 1982). By integrating the promoter into the chromosome, problems with plasmid loss, resulting in an unstable phenotype, could be avoided. TN9 was transformed with either of the linearised plasmids pPGK-GLN1 and pPGK-GLT1, resulting in strains TN15 and TN17,
- 20 respectively. It was verified by PCR analysis that the strong, constitutive promoter of *PGK* had been inserted into the chromosome in front of the structural genes, encoding glutamine synthetase and glutamate synthase, respectively. TN17 was cultivated in non-selective YPD medium for 100 generations in order to remove the resistance against genitacin by homologues recombination of the direct repeats flanking the resistance gene in the chromosome.
- 25 This resulted in isolation of strain TN18 which had lost the genitacin resistance but maintained the strong promoter in front of *GLT1*. TN18 was transformed with pPGK-GLN1 as described above, resulting in strain TN19 with the *PGK* promoter inserted in front of both *GLN1* and *GLT1* in the chromosome. Table 6 lists the phenotypes of all strains that were cultivated in batch reactors in this study.

30

TABLE 6

Strain	Phenotype	Reference
TN1	<i>MAT<math>\alpha</math> ho-<math>\Delta</math>l</i>	Nissen <i>et al.</i> , 1998b
TN9	<i>MAT<math>\alpha</math> ho-<math>\Delta</math>l ura3-<math>\Delta</math>20::SUC2 gdh1-<math>\Delta</math>l::URA3</i>	Nissen <i>et al.</i> , 1998a
TN15	<i>MAT<math>\alpha</math> ho-<math>\Delta</math>l ura3-<math>\Delta</math>20::SUC2 gdh1-<math>\Delta</math>l::URA3 gln1::(PGKp-GLN1)</i>	This study
TN17	<i>MAT<math>\alpha</math> ho-<math>\Delta</math>l ura3-<math>\Delta</math>20::SUC2 gdh1-<math>\Delta</math>l::URA3 glt1::(PGKp-GLT1)</i>	This study
TN19	<i>MAT<math>\alpha</math> ho-<math>\Delta</math>l ura3-<math>\Delta</math>20::SUC2 gdh1-<math>\Delta</math>l::URA3 glt1::(PGKp-GLT1) gln1::(PGKp-GLN1)</i>	This study

Table 6. Phenotypes of the strains cultivated in anaerobic batch fermentations in this study.

- 5 **Glucose limited batch cultivations.** The anaerobic physiology of the genetically engineered *S. cerevisiae* strains were studied in batch cultivations with glucose as the primary carbon source and ammonium nitrogen source. This was done to quantify the effect of the genetic changes on the specific enzyme activities of Gdh1p, Gdh2p, Glt1p and Gln1p, the maximum specific growth rate,  $\mu_{\max}$ , and on the product yields.

10

The specific enzyme activities were measured in protein extracts from biomass samples withdrawn from the bioreactors when the cells were in the exponential growth phase (Table 7).

15 TABLE 7

	TN1	TN9	TN15	TN17	TN19
<b>Gdh1p</b>	1.522	n.d.	n.d.	n.d.	n.d.
<b>Gdh2p</b>	0.020	0.055	0.045	0.028	0.033
<b>Gln1p</b>	0.011	0.009	0.068	0.009	0.062
<b>Glt1p</b>	0.030	0.045	0.045	0.195	0.211

Table 7. Product yields of strains TN1, TN9, TN15, TN17 and TN19 in the anaerobic, glucose-limited batch cultivations that were carried out in this study. Unit: c-moles product per c-mole glucose.

As observed earlier the activity of the NADPH-dependent glutamate dehydrogenases in strain TN1 were 50-100 times higher than the remaining enzymes involved in assimilation of ammonium. This demonstrated the importance of this enzyme in wild-type cells during growth on ammonium as nitrogen source. No activity of Gdh1p could be detected in extracts from the four strains where *GDH1* had been deleted. Thus, GDH3p had no physiological role in this strain background when ammonium was used as nitrogen source. The activity of the NADH-dependent glutamate dehydrogenase, encoded by *GDH2*, was approximately 2.5 times higher in strain TN9 than observed in the haploid wild-type. As described earlier this increase probably was due to a decrease in the intracellular concentration of glutamine since this metabolite represses expression of *GDH2* at the transcriptional level (Nissen *et al.*, 1998a). This decrease could be due to a limitation in the synthesis rate of glutamate in cells with a deletion in *GDH1*. Almost a similar increase in Gdh2p activity was observed in strain TN15, indicating that overexpression of the structural gene for glutamine synthetase only resulted in a limited increase in the intracellular concentration of glutamine. In strains TN17 and TN19 the Gdh2p activity was reduced to a level close to that observed in TN1. Thus, an increase in the intracellular glutamine concentration to wild-type probably was achieved by overexpression of glutamate synthase, resulting in an increase in the synthesis rate of glutamate. Insertion of the strong constitutive promoter of *PGK* in front of *GLN1* into the chromosome of TN15 and TN19 resulted in an increase in the specific activity of glutamine synthetase from approximately 0.010 units per mg total cellular protein (TCP) in strains TN1 and TN9 to 0.068 and 0.062 units per mg TCP, respectively. Insertion of the promoter in front of *GLT1* resulted in a five-fold increase in the activity of glutamate synthase in strains TN17 and TN19 compared to the three other strains. Thus, it was concluded that the new promoter had been inserted correct into the chromosome and that this resulted in the expected increase in the specific activities of glutamine synthetase and glutamate synthase.

In Figure 10 the production of biomass in the exponential growth phases of TN1, TN9, TN17 and TN19 as functions of time are depicted. Deletion of *GDH1* resulted in a reduction in the maximum specific growth rate,  $\mu_{max}$ , from 0.41 h<sup>-1</sup> in strain TN1 to 0.21 h<sup>-1</sup> in strain TN9 (Figure 10). As mentioned earlier this probably was due to a reduction in the synthesis rate of glutamate since the total specific activities of the enzymes that potentially could substitute the physiological role of Gdh1p, Gdh2p and Glt1p, was 15 times lower than the spe-

cific activity of Gdh1p in TN1. Overexpression of *GLN1* in TN9, resulting in strain TN15, gave only a small increase in the maximum specific growth rate, to 0.24 h<sup>-1</sup> (results not shown). This very limited effect of the increase in Gln1p activity was probably due to a slightly higher flux towards synthesis of glutamine. As described earlier glutamine is one of the substrates in the reaction, catalysed by glutamate synthase, wherein glutamate is formed when *GDH1* is deleted. Overexpression of *GLN1* in TN15 probably removes a limitation in the glutamine supply to the reaction catalysed by Glt1p, which results in the observed increase in  $\mu_{\max}$ . Overexpression of *GLT1* had a significant effect on the maximum specific growth rate of TN17. An increase to 0.31 h<sup>-1</sup> was observed. This clearly demonstrated that the reduction in the growth rate of TN9 was caused by a low synthesis rate of glutamate and that this limitation could be partly removed by constructing a strain with a high specific activity of glutamate synthase. Overexpression of both *GLT1* and *GLN1* was obtained in TN19. This led to a further increase in the maximum specific growth rate to 0.37 h<sup>-1</sup>. Thus, the increase in the specific activity of glutamine synthetase had a more pronounced effect on the specific growth rate in a strain where the specific activity of glutamate synthase was high compared to in a strain with a wild-type level of activity. This indicated that overexpression of *GLT1* alone probably resulted in depletion in the intracellular pool of glutamine, which limited the effect of the increase in the specific enzyme activity of Glt1p in TN17. This limitation was apparently removed by overexpression of the structural gene encoding glutamine synthetase.

The consumption of glucose and the production of ethanol, glycerol, acetate, pyruvate, succinate, biomass and carbon dioxide were measured in filtered samples withdrawn from the batch cultivations of TN1, TN9, TN15, TN17 and TN19. No variations between the five strains in formation of the organic acids were detected and thus, only the total yield of these metabolites is listed in Table 8.

TABLE 8

	TN1	TN9	TN15	TN17	TN19
<b>Ethanol</b>	0.480	0.520	0.521	0.530	0.531
<b>Glycerol</b>	0.097	0.060	0.059	0.061	0.060
<b>Biomass</b>	0.121	0.114	0.114	0.110	0.110
<b>Carbon dioxide</b>	0.261	0.275	0.272	0.271	0.273
<b>Organic acids</b>	0.009	0.009	0.010	0.009	0.008
<b>Total</b>	0.968	0.978	0.976	0.981	0.982

5 **Table 8.** The specific activities of the NADPH-dependent and NADH-dependent glutamate dehydrogenases, glutamine synthetase and glutamate synthase in protein extracts from biomass samples withdrawn in the exponential growth phases of strains TN1, TN9, TN15, TN17 and TN19 in the anaerobic, glucose-limited batch cultivations.

10 Figures 11, 12 and 13 show the consumption of glucose and the production of ethanol, glycerol and carbon dioxide as functions of time in the cultivations of strains TN1, TN9 and TN19, respectively. In all cultivations formation of ethanol, glycerol and carbon dioxide stopped immediately after depletion of glucose in the medium. No consumption of the products was detected as long as the bioreactors were sparged with nitrogen, which demonstrated that anaerobic growth conditions had been obtained in the cultivations. The reduction

15 in  $\mu_{\max}$  of TN9 resulted in an increase in the duration of the anaerobic fermentation of the carbon source of 3.5 hours compared to TN1. This elongation of the fermentation time was significantly reduced in strain TN19. Here the fermentation lasted 40 minutes longer than observed in the cultivations of TN1. A relative increase in the ethanol yield of 8% was measured in the cultivations of TN9 compared to TN1 while the relative decrease in the glycerol

20 yield was measured to 38% (Table 8). As described earlier this was due to formation of glutamate by the NADH and ATP consuming reactions catalysed by glutamate synthase and glutamine synthetase in TN9 compared to formation of glutamate by the NADPH consuming reaction catalysed by glutamate dehydrogenase I in TN1. The increase in ATP consumption in biomass synthesis resulted in a reduction of the biomass yield in TN9, by 6%.

25 Overexpression of *GLN1* alone had no significant influence on the product formation of strain TN15 compared to TN9. The five-fold increase in the specific glutamate synthase

*E. coli* DH5 $\alpha$  and *L. lactis* subsp. *cremoris* were both transformed with pTRKH2-p170-cth and transformants were selected on plates containing complex medium (LB and GM13, respectively) supplemented with erythromycin. Independent pTRKH2-p170-cth transformants of both *E. coli* and *L. lactis* were grown in shake flasks in LB medium and GM13 medium, respectively, supplemented with erythromycin. In the late exponential growth phase, cell

samples were withdrawn from the shake flasks and the protein pools of the cells were extracted.

The extracted protein pools were assayed for activity of the cytoplasmic transhydrogenase.

5 The results are listed below

Microorganism	Transhydrogenase activity (U per mg protein)
<i>E. coli DH5<math>\alpha</math></i>	not detectable
<i>E. coli DH5<math>\alpha</math></i> pTRKH2-p170-cth	0.568
<i>L. lactis</i> subsp. <i>cremoris</i>	not detectable
<i>L. lactis</i> subsp. <i>cremoris</i> pTRKH2-p170-cth	0.107

From the data it was concluded that the cytoplasmic transhydrogenase had been successfully  
10 expressed in both *E. coli* and *L. lactis*.





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## Patent claims

**1. Recombinant microbial cell comprising**

- 5 i) a first enzyme activity controlling assimilation of a nitrogen nutrient source,

wherein the first enzyme activity is encoded by a first nucleic acid operable linked to an expression signal not natively associated with the first nucleic acid, and

wherein the expression of the first enzyme activity is increased as compared to the expression of the first enzyme activity when the first nucleic acid is associated with its native expression signal,

**and/or**

- ii) a second enzyme activity controlling assimilation of a nitrogen nutrient source,

wherein the second enzyme activity is encoded by a second nucleic acid operable linked to an expression signal not natively associated with the second nucleic acid, and

25 wherein the expression of the second enzyme activity is increased as compared to the expression of the second enzyme activity when the second nucleic acid is associated with its native expression signal,

the cell further comprising

- iii) a reduced or eliminated expression of a third enzyme activity encoded by a third nucleic acid and controlling assimilation in the cell of a nitrogen nutrient source.

wherein the expression of the third enzyme activity is reduced or eliminated as compared to the expression of the third enzyme activity when the third nucleic acid is associated with its native expression signal.

- 5      2. Microbial cell according to claim 1, the cell comprising
- i)      a further enzyme activity, the further enzyme activity mediates an energy  
              yielding first reaction resulting in a production of a first metabolite,  
              wherein
- 10      ii)      the first reaction being operably linked to an energy requiring second re-  
              action resulting in assimilation of a nutrient source.
- 15      3. Microbial cell according to claim 2, wherein the energy requiring second reaction  
              resulting in assimilation of a nutrient source is controlled at least by the first  
              and/or second enzyme activity.
- 20      4. Microbial cell according to claim 1, the cell comprising
- i)      a further enzyme activity, wherein the further enzyme activity mediates  
              an energy yielding first reaction resulting in a production of a first me-  
              tabolite, wherein
- 25      ii)      the further enzyme activity, when expressed at an increased level, re-  
              sults in an increased production of the first metabolite, wherein
- 30      iii)      the increased expression of the further enzyme activity and/or the in-  
              creased production of the first metabolite is operably linked to an in-  
              creased expression of the first and/or second enzyme activity.
- 35      5. Microbial cell according to claim 1, the cell being selected from the group con-  
              sisting of a fungal cell, a yeast cell, and a bacterial cell.
6. Microbial cell according to claim 6, the cell being a yeast cell.

7. Microbial cell according to claim 1, wherein the nitrogen source is ammonia and/or an ammonium ion.
- 5 8. Microbial cell according to claim 1 wherein the first or second enzyme activity is mediating at least one enzymatic reaction resulting in the assimilation of ammonia and/or an ammonium ion in the microbial cell.
- 10 9. Microbial cell according to claim 1 wherein the first and second enzyme activities are each mediating at least one enzymatic reaction resulting in the assimilation of ammonia in the microbial cell.
10. Microbial cell according to claim 1 wherein at least one of the first and second enzyme activities is mediating a biosynthetic reaction.
- 15 11. Microbial cell according to claim 1 wherein the first enzyme activity is a glutamate synthase activity.
- 20 12. Microbial cell according to claim 11 wherein the activity is a *Saccharomyces cerevisiae* glutamate synthase, or a functionally equivalent activity capable of catalysing a glutamate synthase reaction.
- 25 13. Microbial cell according to claim 12 wherein the activity is that encoded by *GLT1* of *Saccharomyces cerevisiae* as deposited under DSM Accession Number 12275.
14. Microbial cell according to claim 1 wherein the second enzyme activity is a glutamine synthetase activity.
- 30 15. Microbial cell according to claim 14 wherein the activity is a *Saccharomyces cerevisiae* glutamine synthetase activity, or a functionally equivalent activity capable of catalysing a glutamine synthetase reaction.
- 35 16. Microbial cell according to claim 15 wherein the activity is that encoded by *GLN1* of *Saccharomyces cerevisiae* as deposited under DSM Accession Number 12274.

17. Microbial cell according to claim 1 wherein the third enzyme activity is a glutamate dehydrogenase activity.
- 5 18. Microbial cell according to claim 17 wherein the activity is a *Saccharomyces cerevisiae* glutamate dehydrogenase activity, or a functionally equivalent activity capable of catalysing a glutamate dehydrogenase reaction.
- 10 19. Microbial cell according to claim 18 wherein the activity is that encoded by *GDH1* of *Saccharomyces cerevisiae*.
20. Microbial cell according to claim 1 wherein the expression of the third enzyme activity is reduced by at least 50%.
- 15 21. Microbial cell according to claim 1 wherein the third enzyme activity is not expressed.
22. Microbial cell according to claim 1 wherein the third enzyme activity has been eliminated.
- 20 23. Microbial cell according to claim 21 or 22 wherein a nucleotide sequence encoding the third enzyme activity and/or an expression signal directing expression of the activity has been partly or wholly deleted from a chromosomal replicon and/or an extrachromosomal replicon harboured by the microbial cell.
- 25 24. Microbial cell according to any of claims 20 to 23 wherein the third enzyme activity is a glutamate dehydrogenase activity.
- 30 25. Microbial cell according to claim 24 wherein the glutamate dehydrogenase activity is that encoded by *GDH1* of *Saccharomyces cerevisiae*, or a functionally equivalent activity capable of catalysing a glutamate dehydrogenase reaction.
- 35 26. Microbial cell according to claim 25, the cell being *Saccharomyces cerevisiae* TN19 as deposited under Accession Number DSM 12276.



27. Microbial cell according to claim 25, the cell being *Saccharomyces cerevisiae* TN22 as deposited under Accession Number DSM 12277.
- 5 28. Microbial cell according to any of claims 20 to 27 wherein the expression of the first or second enzyme activity is increased by a factor of at least 1.5.
29. Microbial cell according to any of claims 20 to 28 wherein the expression of the first and second enzyme activities are increased by a factor of at least 1.5.
- 10 30. Microbial cell according to any of the previous claims and further comprising a fourth nucleic acid encoding a fourth enzyme activity controlling an intracellular redox system of the cell, wherein the fourth nucleic acid is operably linked to an expression signal not natively associated with the fourth nucleic acid.
- 15 31. Microbial cell according to claim 30 wherein the fourth enzyme activity is an intracellular transhydrogenase activity.
32. Microbial cell according to claim 30 wherein the transhydrogenase activity is that encoded by *CTH* of *Azotobacter vinelandii* as harboured by *Saccharomyces*  
20 *cerevisiae* TN4 deposited under DSM Accession Number 12267.
33. Microbial cell according to any of the previous claims in the form of a frozen or freeze-dried preparation such as a lyophilisate.
- 25 34. Composition comprising the microbial cell according to any of the previous claims and a carrier.
35. Composition according to claim 34 wherein the carrier is a physiologically acceptable carrier such as a water-based liquid, preferably a broth suitable for  
30 culturing the microbial cell.
36. Composition according to claim 34 or 35, wherein the composition is a fermentation starter culture.

37. Composition according to any of claims 34 to 36 for use in the production of a first metabolite.

5 38. Microbial cell according to any of claims 1 to 33 for use in the production of a first metabolite.

10 39. Microbial cell according to claim 38, wherein the increased expression of the first and/or second enzyme activity encoded by the first and/or second nucleic acid, respectively, results in an increased production of a first metabolite, the production being increased as compared to the production of the metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.

15 40. Microbial cell according to claim 39, wherein the production of the first metabolite is increased by a factor of at least 1.10.

41. Microbial cell according to claim 38 wherein the microbial cell is a yeast cell.

20 42. Microbial cell according to claim 38 wherein the first metabolite is ethanol.

25 43. Microbial cell according to claim 41, wherein the yeast cell further produces a second metabolite, the production of the second metabolite being decreased as compared to the production of the metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.

44. Microbial cell according to claim 43 wherein the second metabolite is glycerol.

30 45. Microbial cell according to any of claims 1 to 33 and 38 to 44, or the composition according to any of claims 34 to 37, for use in a preparation of a drinkable or an edible product.

35 46. Microbial cell according to any of claims 1 to 33 and 38 to 44, or the composition according to any of claims 34 to 37, for use in a production of a first metabolite for use in a drinkable or an edible product.

47. Microbial cell according to claim 46 wherein the first metabolite provides a desirable organoleptic quality to the product.
48. Microbial cell according to claim 46, wherein the metabolite is ethanol.
- 5
49. Use of the microbial cell according to any of claims 1 to 33 and 38 to 44, or the composition according to any of claims 34 to 37, in a production of a first metabolite.
- 10
50. Use of claim 49, wherein the production of the first metabolite is increased in a cell wherein the expression of the first and/or second enzyme activity encoded by the first and/or second nucleic acid, respectively, is increased as compared to the production of the first metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.
- 15
51. Use of claim 49, wherein the microbial cell is a yeast cell.
52. Use of claim 49, wherein the first metabolite is ethanol.
- 20
53. Use according to claim 51 or 52, wherein the yeast cell further produces a second metabolite, the production of the second metabolite is decreased as compared to the production of the second metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.
- 25
54. Use according to claim 53 wherein the second metabolite is glycerol.
55. Use according to claim 54 in the preparation of a drinkable or an edible product.
- 30
56. Method of producing a first metabolite, the method comprising the steps of
- i) cultivating the microbial cell according to any of claims 1 to 33 and 38 to 44, or the composition according to any of claims 34 to 37, in a suitable growth medium and under such conditions that the microbial cell is producing a first metabolite, and optionally
- 35

- ii) isolating the first metabolite in a suitable form, and further optionally
- iii) purifying the isolated first metabolite.

5 57. Method of claim 56, wherein the production of the first metabolite is increased in a cell wherein the expression of the first and/or second enzyme activity encoded by the first and/or second nucleic acid, respectively, is increased, as compared to the production of the first metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.

10

58. Method of claim 57, wherein the production of the metabolite is increased by a factor of at least 1.02, such as 1.04, for example 1.08, such as 1.16, for example 1.25, such as 1.4.

15

59. Method of claim 56 wherein the microbial cell is a yeast cell.

60. Method of claim 56 wherein the first metabolite is ethanol.

20

61. Method of claim 56, wherein the yeast cell further produces a second metabolite, the production of the second metabolite is decreased as compared to the production of the second metabolite in a cell wherein the first and/or second nucleic acid is associated with a native expression signal.

25

62. Method of claim 61 wherein the second metabolite is glycerol.

63. Method of constructing a microbial cell according to any of claims 1 to 33, and 38 to 48, the method comprising the steps of

30

- i) operably linking a nucleotide sequence encoding the first enzyme activity with an expression signal not natively associated with the first nucleotide sequence, wherein the expression of the first enzyme activity is increased as compared to the expression of the first enzyme activity when the first nucleic acid is associated with its native expression signal, and/or

35

- 30 67. Method of claim 66, wherein the glutamate synthase activity is that encoded by *GLT1* of *Saccharomyces cerevisiae* TN17 as deposited under DSM Accession Number 12275, or a functionally equivalent activity capable of catalysing a glutamate synthase reaction.

68. Method of claim 64 wherein the second enzyme activity is a glutamine synthetase activity.

5 69. Method of claim 68 wherein the glutamine synthetase activity is that encoded by *GLN1* of *Saccharomyces cerevisiae* TN15 as deposited under DSM Accession Number 12274, or a functionally equivalent activity capable of catalysing a glutamine synthetase reaction.

10 70. Method of claim 64 wherein the third enzyme activity, when present in the microbial cell, is a glutamate dehydrogenase activity, preferably one encoded by *GDH1* of *Saccharomyces cerevisiae*.

15 71. Method of claim 64 wherein the cell is *Saccharomyces cerevisiae* TN19 as deposited under DSM Accession Number 12276.

72. Method of any of claims 63 to 71, the method comprising a further step of freezing or freeze-drying the microbial cell in the preparation of a reconstitutable lyophilisate.



FIGURE 1

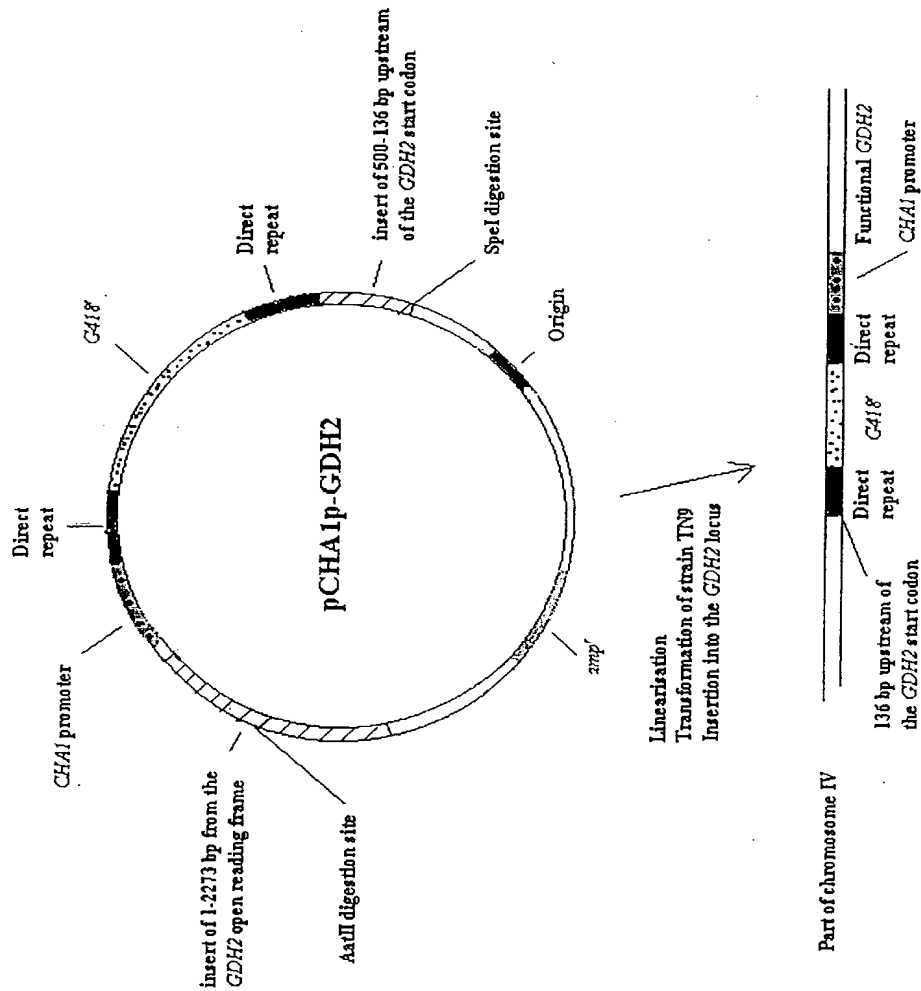




FIGURE 2

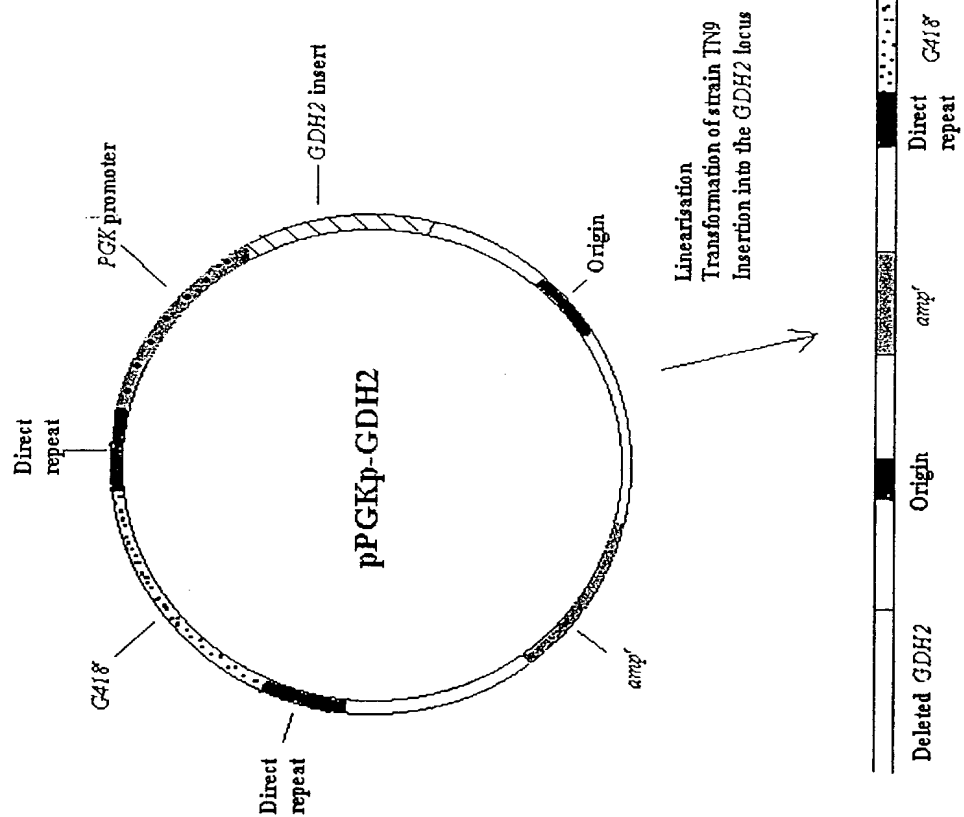


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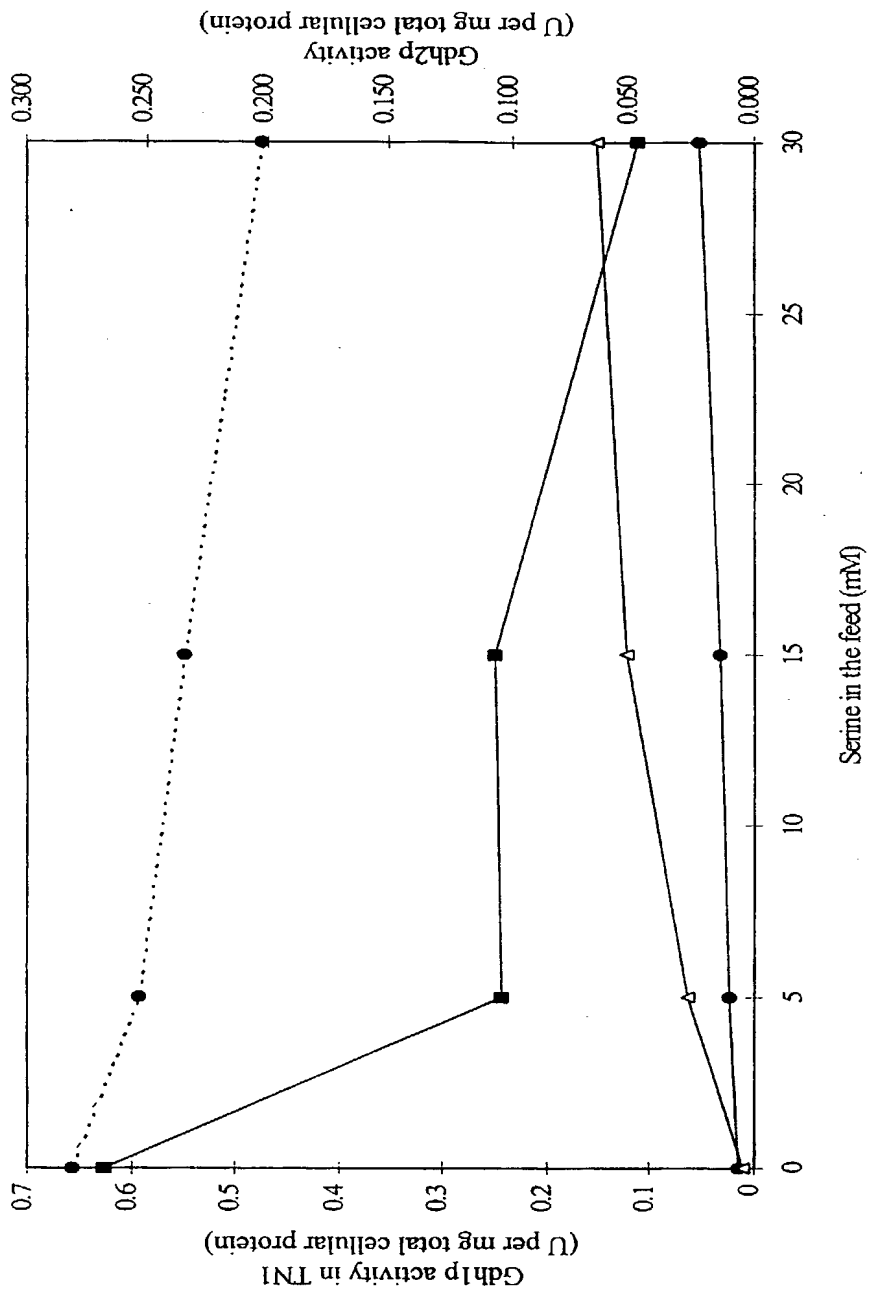


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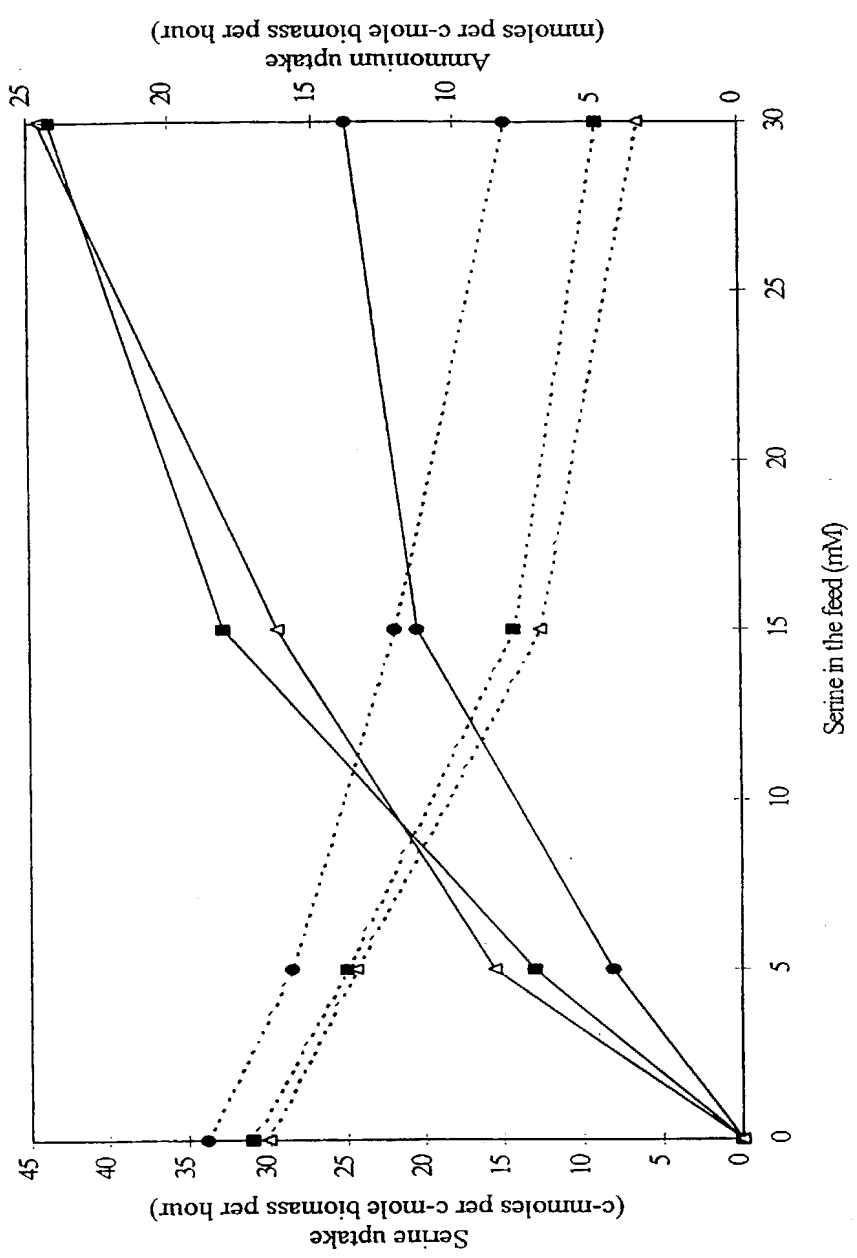


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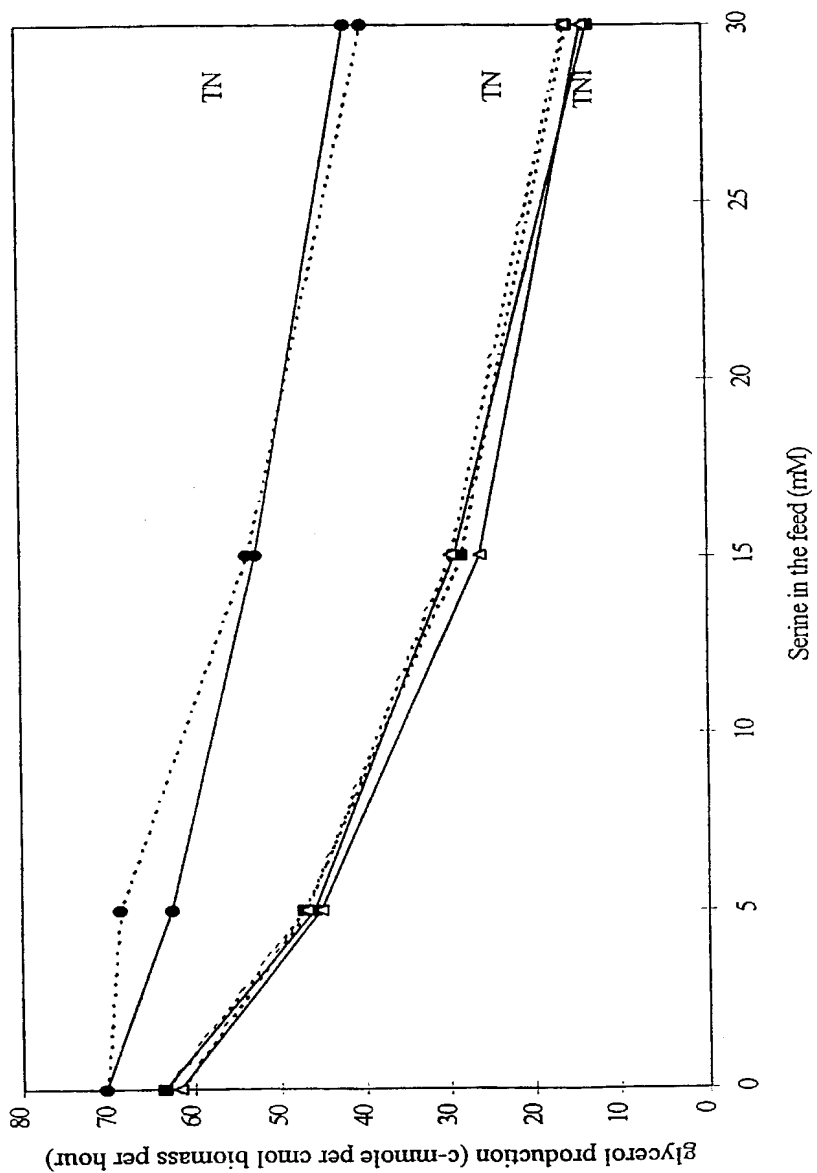
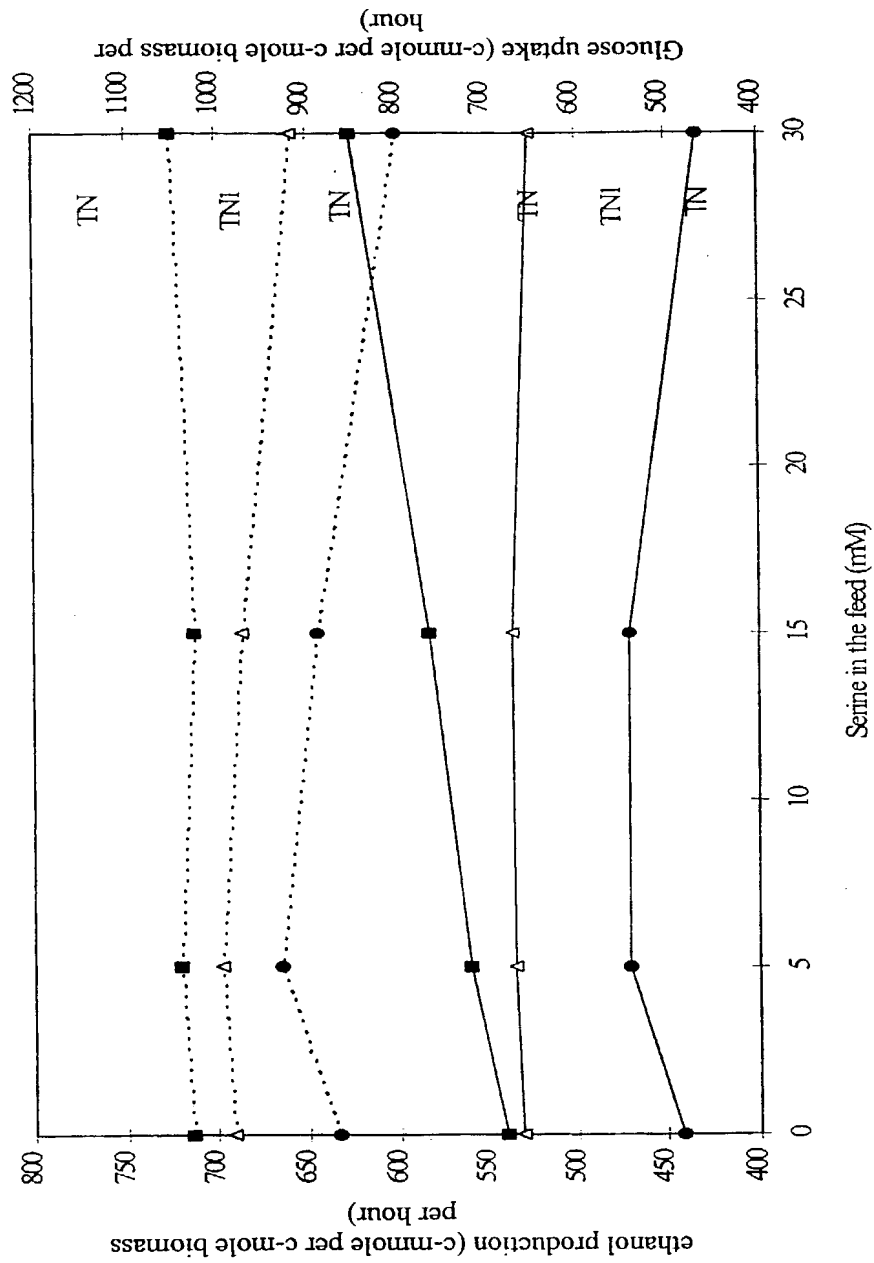


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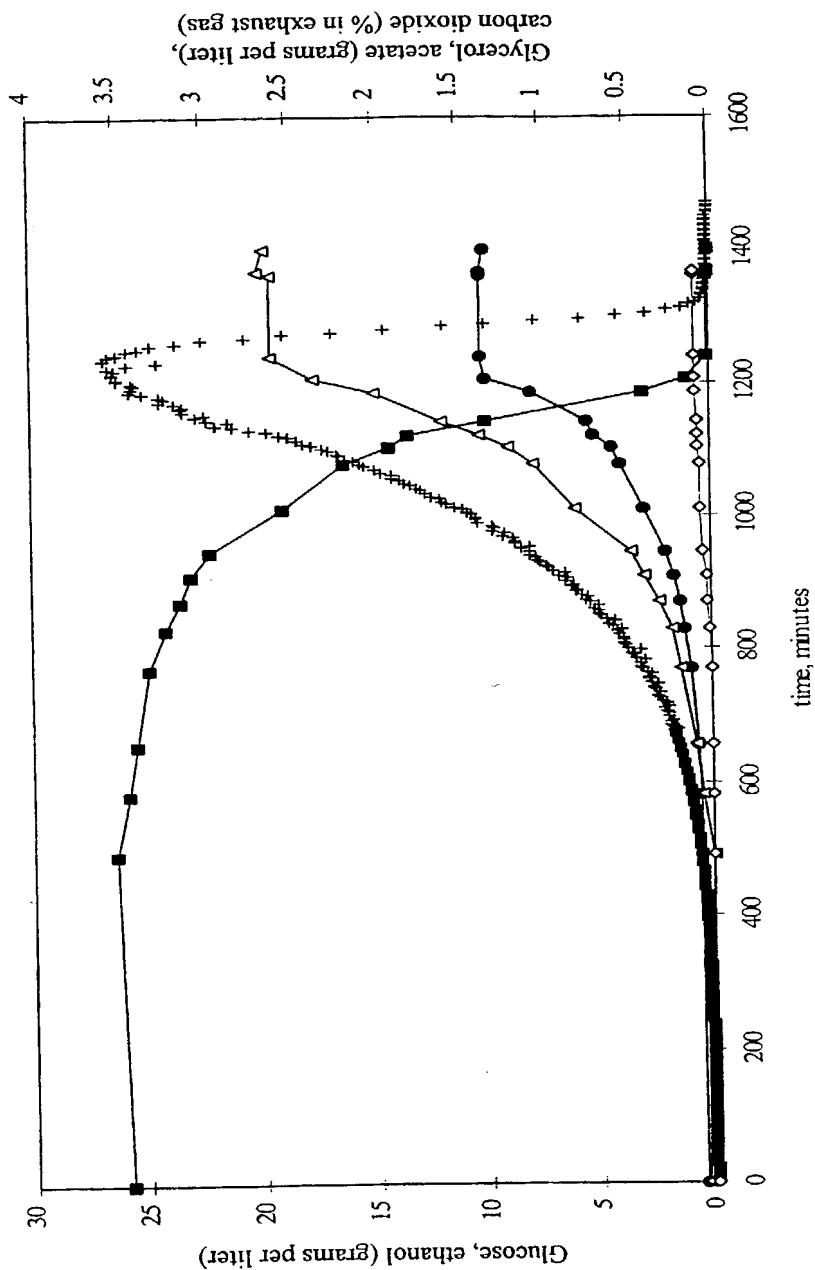
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FIGURE 7



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FIGURE 8

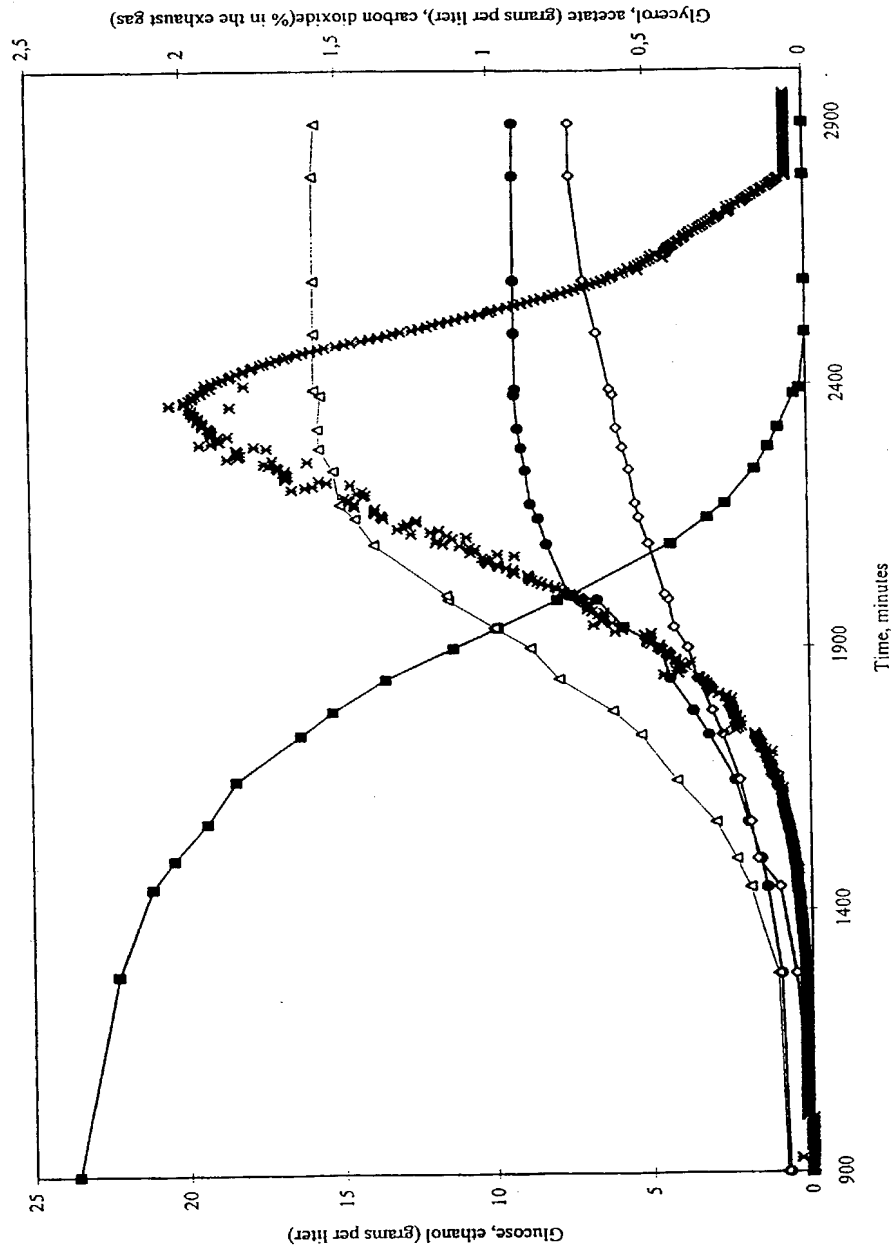
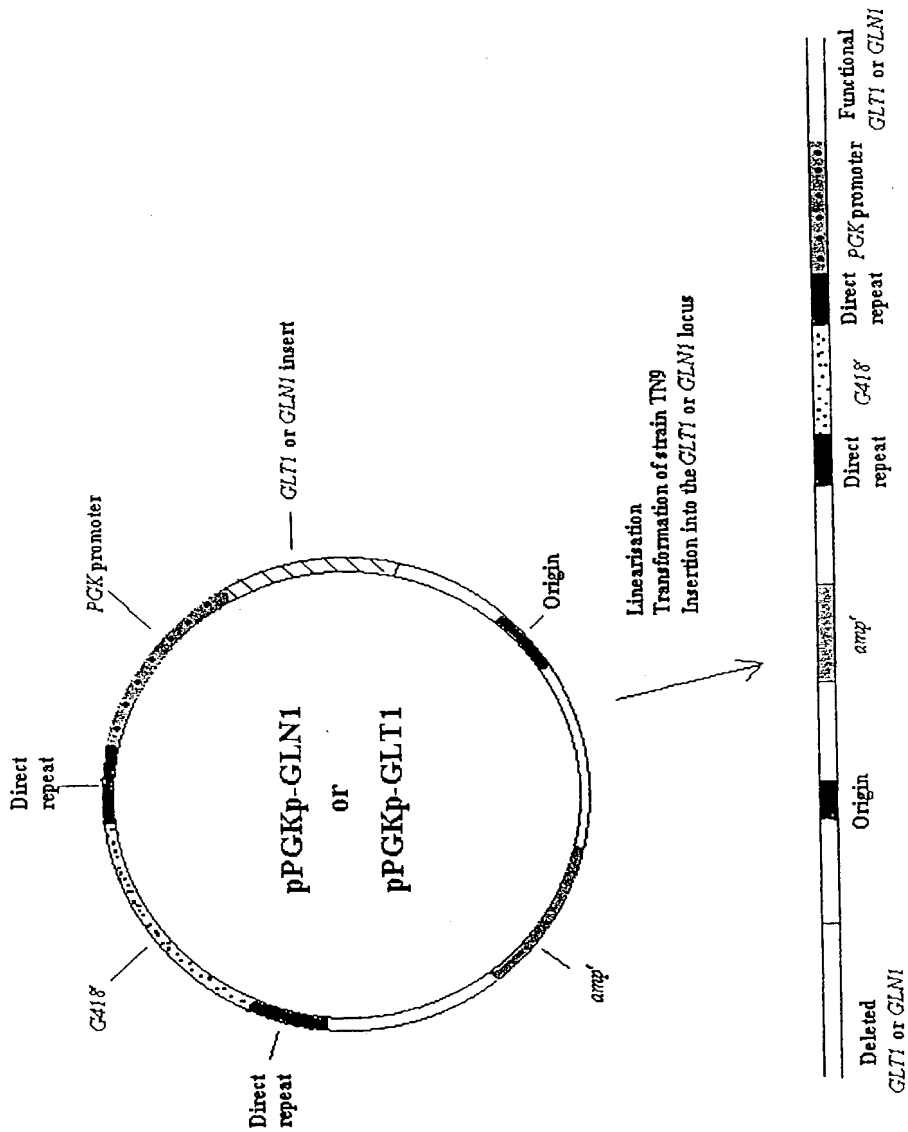


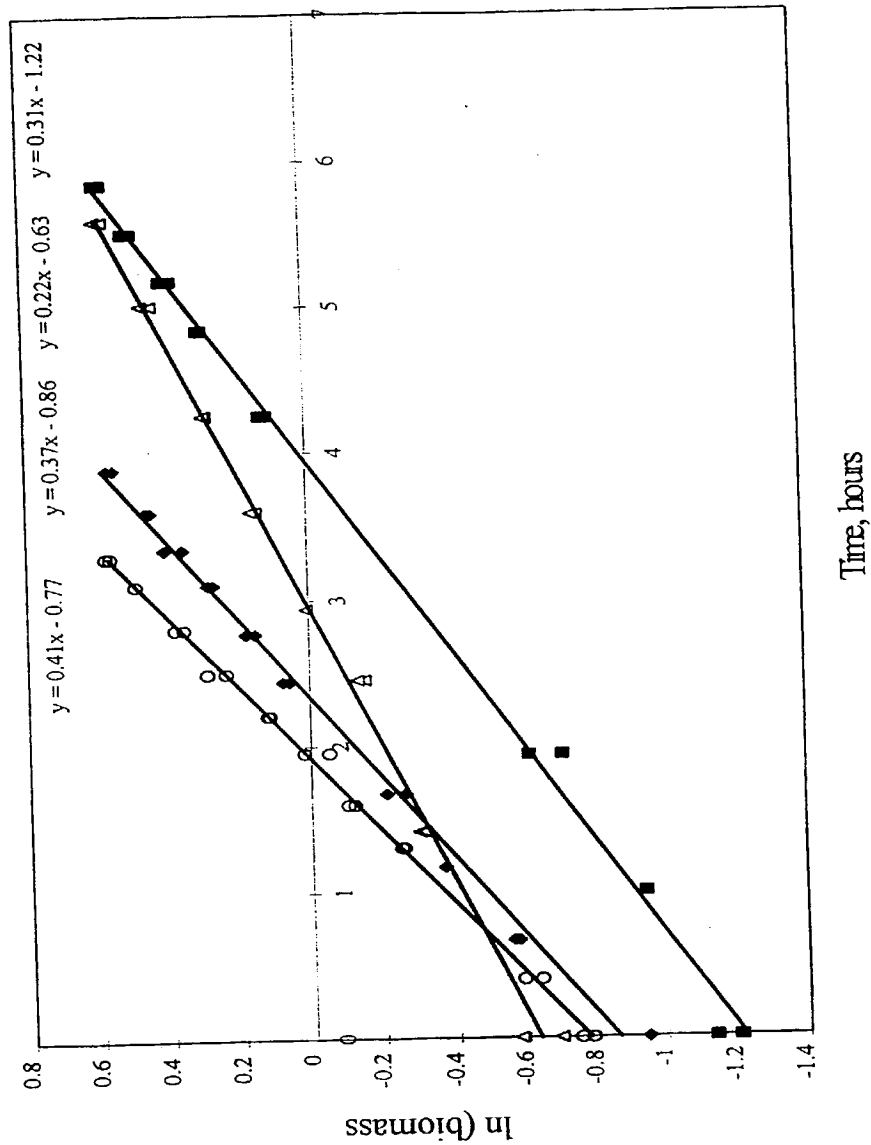
FIGURE 9





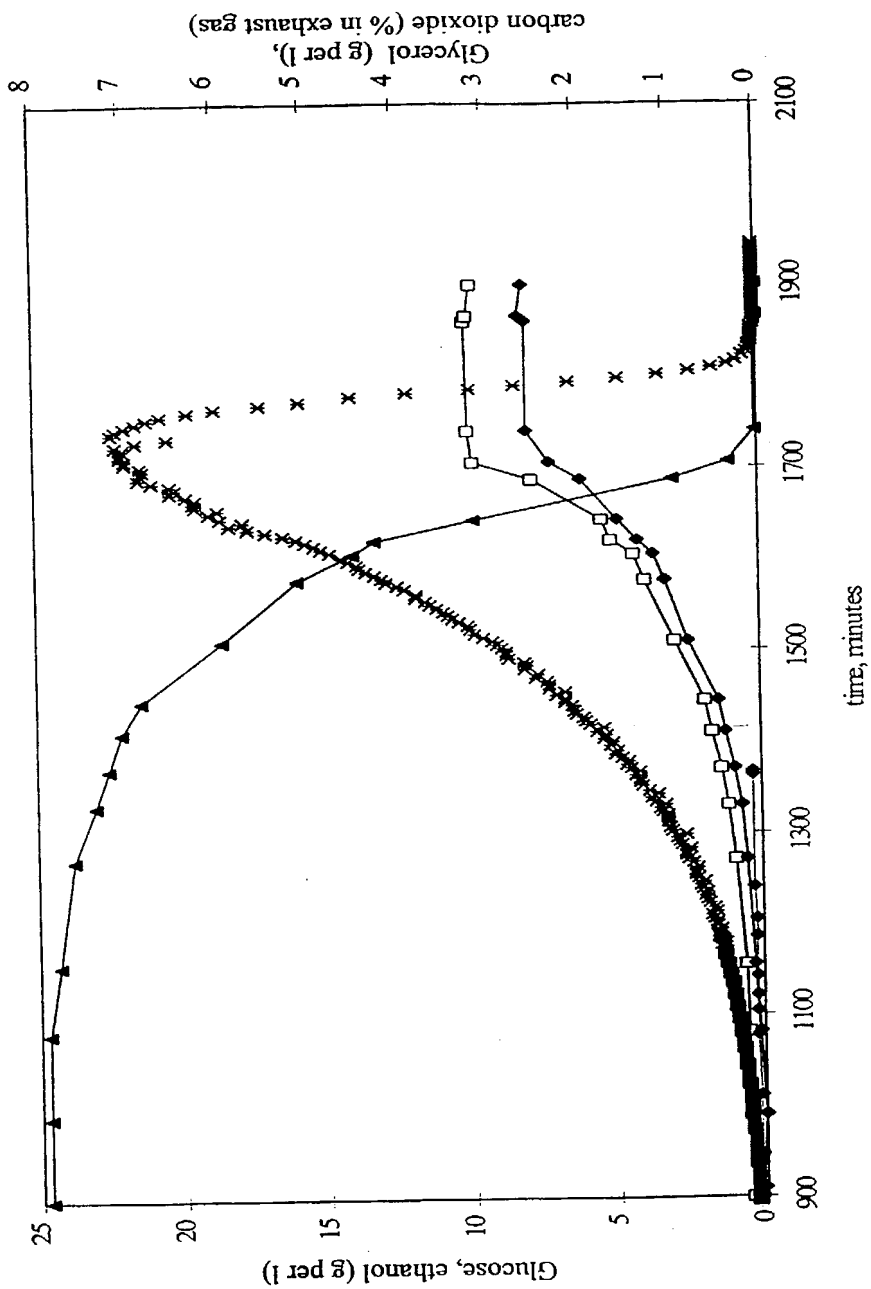
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FIGURE 10



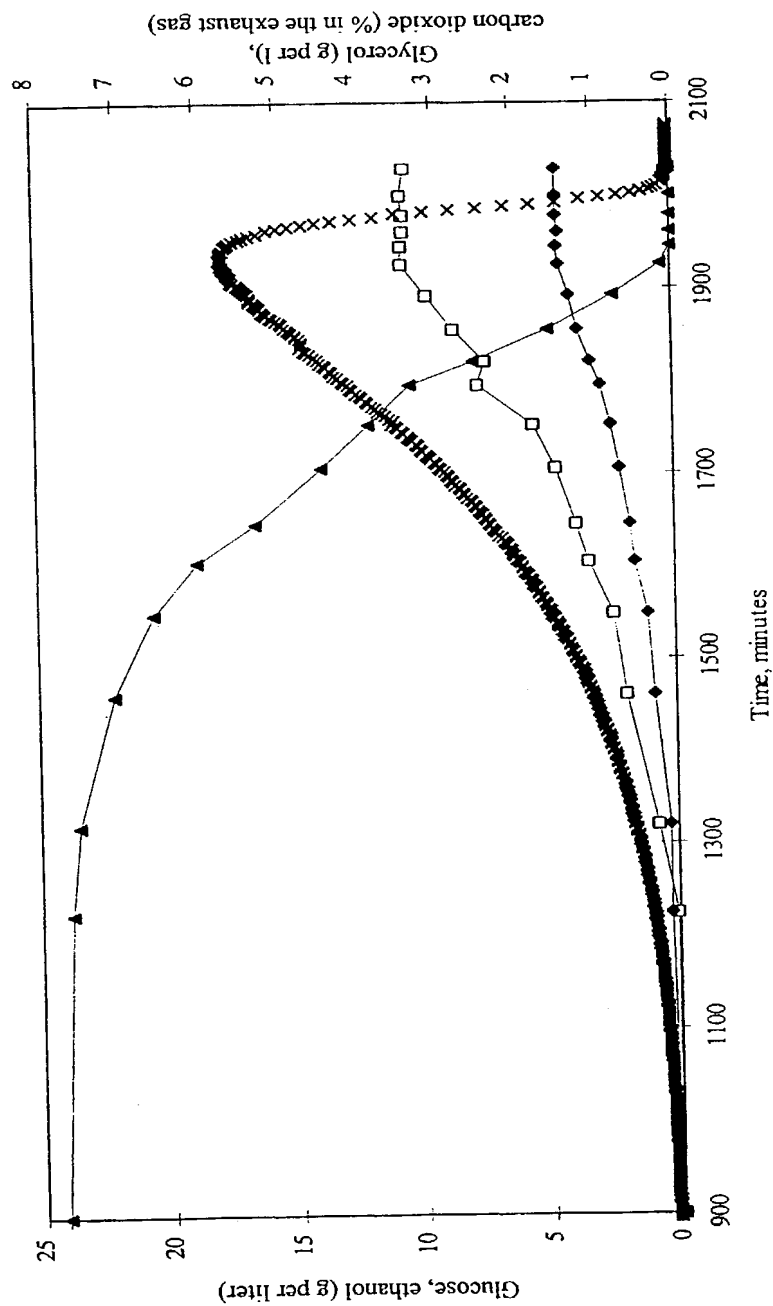
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FIGURE 11



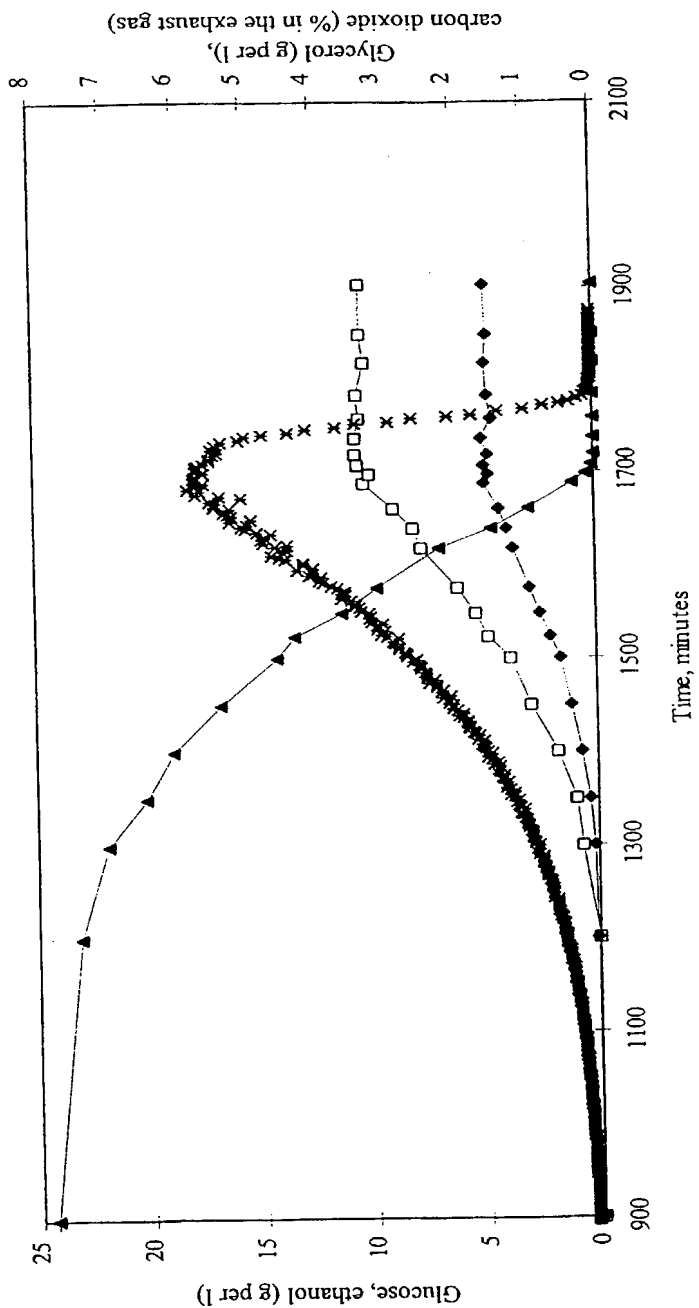
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FIGURE 12



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FIGURE 13



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[X] Original [ ] Substitute [ ] Supplemental

Atty. Docket: NIELSEN4

**Combined Declaration for Patent Application and Power of Attorney**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**METABOLICALLY ENGINEERED MICROBIAL CELL WITH AN ALTERED METABOLITE PRODUCTION**

the specification of which (check one)

☐ is attached hereto;☐ was filed in the United States under 35 U.S.C. §111 on \_\_\_\_\_, as U.S. Appl. No. \_\_\_\_\_\*, or☒ was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an international (PCT) application, PCT/DK99/00397 filed July 12, 1999, entry requested on January 10, 2001\*; national stage application received U.S. Appl. No. 09/743,414; §371/§102(e) date \_\_\_\_\_\* (\* if known)

and was amended on January 10, 2001 \_\_\_\_\_ (if applicable).

(include dates of amendments under PCT Art. 19 and 34 if PCT)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119 (a)-(d) and 365 (b) of any prior foreign application(s) for patent or inventor's certificate, or §365(a) of any prior PCT application(s) designating a country other than the U.S., listed below with the "Yes" box checked, and have also identified below, by checking the "No" box, any foreign application for patent or inventor's certificate or PCT international application having a filing date before that of the application on which priority is claimed:

FA 1998 00967	DENMARK	10 July 1998	<input checked="" type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day Month Year Filed)	YES	NO
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day Month Year Filed)	YES	NO

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional applications listed below:

(Application No.)	(Day Month Year Filed)
_____	_____
(Application No.)	(Day Month Year Filed)
_____	_____

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or under §365(c) of any prior PCT international application(s) designating the U.S., listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT international application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
_____	_____	_____
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
_____	_____	_____

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

All of the practitioners associated with Customer Number 001444

Direct all correspondence to the address associated with Customer Number 001444, which is presently:

**BROWDY AND NEIMARK, P.L.L.C.**  
624 Ninth Street, N.W.  
Washington, D.C. 20001-5303  
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The undersigned hereby authorizes the U.S. Attorneys or Agents appointed herein to accept and follow instructions from HOIBERG APS as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

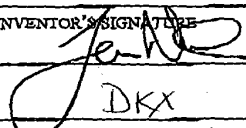
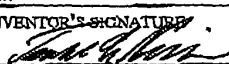

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Atty. Docket: NIELSEN4

Title: METABOLICALLY ENGINEERED MICROBIAL CELL...U.S. Application filed \_\_\_\_\_, Serial No. 09/743,413PCT Application filed July 12, 1999, Serial No. PCT/DK99/00397

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00	FULL NAME OF FIRST INVENTOR <u>Jens NIELSEN</u>	INVENTOR'S SIGNATURE 	DATE <u>30/4/01</u>
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	POST OFFICE ADDRESS <u>Hans Bruuns Vej 8, DK-2920 Charlottenlund, DENMARK</u>		
2-00	FULL NAME OF SECOND JOINT INVENTOR <u>Torben Lausgaard NISSEN</u>	INVENTOR'S SIGNATURE 	DATE <u>30/4-2001</u>
	RESIDENCE <u>Frederiksberg, DENMARK</u>	CITIZENSHIP <u>DKX</u>	DENMARK
	POST OFFICE ADDRESS <u>Asmussens Allé 5, 4.tv., DK-1808 Frederiksberg C, DENMARK</u>		
3-00	FULL NAME OF THIRD JOINT INVENTOR <u>Morten C. KIBLAND-BRANDT</u>	INVENTOR'S SIGNATURE 	DATE <u>30 APR 01</u>
	RESIDENCE <u>Copenhagen, DENMARK</u>	CITIZENSHIP <u>DKX</u>	DENMARK
	POST OFFICE ADDRESS <u>Lundbyesgade 3, DK-1771 Copenhagen V, DENMARK</u>		
	FULL NAME OF FOURTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
	RESIDENCE	CITIZENSHIP	
	POST OFFICE ADDRESS		
	FULL NAME OF FIFTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
	RESIDENCE	CITIZENSHIP	
	POST OFFICE ADDRESS		
	FULL NAME OF SIXTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
	RESIDENCE	CITIZENSHIP	
	POST OFFICE ADDRESS		
	FULL NAME OF SEVENTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
	RESIDENCE	CITIZENSHIP	
	POST OFFICE ADDRESS		

ALL INVENTORS MUST REVIEW APPLICATION AND DECLARATION BEFORE SIGNING. ALL ALTERATIONS MUST BE INITIALED AND DATED BY ALL INVENTORS PRIOR TO EXECUTION. NO ALTERATIONS CAN BE MADE AFTER THIS DECLARATION IS SIGNED. ALL PAGES OF DECLARATION MUST BE SEEN BY ALL INVENTORS.

## SEQUENCE LISTING

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Nissen, Torben L

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agc tgg cgt ttc gtc gac gac gtg ccg acc ggc atc tac acc att ccg 1056
Ser Trp Arg Phe Val Asp Asp Val Pro Thr Gly Ile Tyr Thr Ile Pro
340 345 350

gag atc agt tcg gtc ggc aag acc gag cgc gaa ctg acc cag gcg aag 1104
Glu Ile Ser Ser Val Gly Lys Thr Glu Arg Glu Leu Thr Gln Ala Lys
355 360 365

gtt ccc tac gag gtc ggc aag gcc ttc ttc aag ggc atg gcc cgg gca 1152
Val Pro Tyr Glu Val Gly Lys Ala Phe Phe Lys Gly Met Ala Arg Ala
370 375 380

cag atc gcc gtc gag aag gcc ggc atg ctg aag atc ctc ttt cac cgc 1200
Gln Ile Ala Val Glu Lys Ala Gly Met Leu Lys Ile Leu Phe His Arg
385 390 395 400

gag acg ctg gaa atc ctc ggc gtg cac tgc ttc ggc tat cag gct tcg 1248
Glu Thr Leu Glu Ile Leu Gly Val His Cys Phe Gly Tyr Gln Ala Ser
405 410 415

gaa atc gtc cat atc ggc cag gcg atc atg aac cag aag ggc gag gcg 1296
Glu Ile Val His Ile Gly Gln Ala Ile Met Asn Gln Lys Gly Glu Ala
420 425 430

aat acc ctc aag tat ttc atc aac acc acc ttc aac tac ccg acc atg 1344
Asn Thr Leu Lys Tyr Phe Ile Asn Thr Thr Phe Asn Tyr Pro Thr Met
435 440 445

gcc gag gcc tac cgg gtg gcg gcc tac gac ggt ctc aat cgg ctt ttt 1392
Ala Glu Ala Tyr Arg Val Ala Ala Tyr Asp Gly Leu Asn Arg Leu Phe
450 455 460

tga 1395

465

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 <212> PRT  
 <213> *Azotobacter vinelandii*

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 Gly Glu Gly Ala Ala Met Asn Ala Val Lys Ala Gly Arg Lys Val Ala  
 20 25 30

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Thr	Ile 50	Pro	Ser	Lys	Ala	Leu 55	Arg	His	Ser	Val	Arg 60	Gln	Ile	Met	Gln
Tyr	Asn 65	Asn	Asn	Pro	Leu 70	Phe	Arg	Gln	Ile	Gly 75	Glu	Pro	Arg	Trp	Phe 80
Ser	Phe	Ala	Asp	Val 85	Leu	Lys	Ser	Ala	Glu 90	Gln	Val	Ile	Ala	Lys 95	Gln
Val	Ser	Ser	Arg 100	Thr	Gly	Tyr	Tyr	Ala 105	Arg	Asn	Arg	Ile	Asp 110	Thr	Phe
Phe	Gly	Thr 115	Ala	Ser	Phe	Cys	Asp 120	Glu	His	Thr	Ile	Glu 125	Val	Val	His
Leu	Asn 130	Gly	Met	Val	Glu	Thr 135	Leu	Val	Ala	Lys	Gln 140	Phe	Val	Ile	Ala
Thr 145	Gly	Ser	Arg	Pro	Tyr 150	Arg	Pro	Ala	Asp	Val 155	Asp	Phe	Thr	His	Pro 160
Arg	Ile	Tyr	Asp	Ser 165	Asp	Thr	Ile	Leu	Ser 170	Leu	Gly	His	Thr	Pro 175	Arg
Arg	Leu	Ile	Ile 180	Tyr	Gly	Ala	Gly	Val 185	Ile	Gly	Cys	Glu	Tyr 190	Ala	Ser
Ile	Phe	Ser 195	Gly	Leu	Gly	Val	Leu 200	Val	Asp	Leu	Ile	Asp 205	Asn	Arg	Asp
Gln	Leu 210	Leu	Ser	Phe	Leu	Asp 215	Asp	Glu	Ile	Ser	Asp 220	Ser	Leu	Ser	Tyr
His 225	Leu	Arg	Asn	Asn	Asn 230	Val	Leu	Ile	Arg 235	His	Asn	Glu	Glu	Tyr	Glu 240
Arg	Val	Glu	Gly	Leu 245	Asp	Asn	Gly	Val	Ile 250	Leu	His	Leu	Lys	Ser 255	Gly
Lys	Lys	Ile	Lys 260	Ala	Asp	Ala	Phe	Leu 265	Trp	Ser	Asn	Gly	Arg 270	Thr	Gly
Asn	Thr	Asp 275	Lys	Leu	Gly	Leu	Glu 280	Asn	Ile	Gly	Leu	Lys 285	Ala	Asn	Gly
Arg	Gly 290	Gln	Ile	Gln	Val	Asp 295	Glu	His	Tyr	Arg	Thr 300	Glu	Val	Ser	Xaa
Ile 305	Tyr	Ala	Ala	Gly	Asp 310	Val	Ile	Gly	Trp	Pro 315	Ser	Leu	Ala	Ser	Ala 320
Ala	Tyr	Asp	Gln	Gly 325	Arg	Ser	Ala	Ala	Gly 330	Ser	Ile	Thr	Glu	Asn 335	Asp

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<220>  
<223> Description of Artificial Sequence: Primer

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<223> Description of Artificial Sequence: Primer

<400> 6  
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<212> DNA  
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<220>  
<223> Description of Artificial Sequence: Primer

<400> 14  
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RECEIVED PTO 03 OCT 2001 #6

SEQUENCE LISTING

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Nissen, Torben L  
Kielland-Brandt, Morten C

<120> Metabolically engineered microbial cell with an altered  
metabolite production

<130> NIELSEN=4

<140> 09/743,414

<141> 2001-01-10

<150> PCT/DK99/00397

<151> 1999-07-12

<150> PA 1998 00967

<151> 1998-07-10

<160> 14

<170> PatentIn Ver. 2.1

<210> 1

<211> 1395

<212> DNA

<213> Azotobacter vinelandii

<220>

<221> CDS

<222> (1)..(1395)

<223>

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Gly Glu Gly Ala Ala Met Asn Ala Val Lys Ala Gly Arg Lys Val Ala  
20 25 30

gtc gtg gat gat cgc ccc cag gtc ggc ggc aac tgc acc cac ctc gga 144  
Val Val Asp Asp Arg Pro Gln Val Gly Gly Asn Cys Thr His Leu Gly  
35 40 45

acc att ccc tcc aag gcg ctg cgc cac tcg gtg cgg cag atc atg cag 192  
Thr Ile Pro Ser Lys Ala Leu Arg His Ser Val Arg Gln Ile Met Gln  
50 55 60

tac aac aac aat ccg ctg ttc cgc cag atc ggc gag ccg cgc tgg ttt 240  
Tyr Asn Asn Asn Pro Leu Phe Arg Gln Ile Gly Glu Pro Arg Trp Phe  
65 70 75 80

tcc ttc gcc gat gtc ctg aag agc gcc gag cag gtc atc gcc aag cag 288  
Ser Phe Ala Asp Val Leu Lys Ser Ala Glu Gln Val Ile Ala Lys Gln  
85 90 95

gtt	tcc	tcg	cgg	acc	ggc	tac	tat	gcg	cgc	aac	cgt	atc	gat	acc	ttc	336
Val	Ser	Ser	Arg	Thr	Gly	Tyr	Tyr	Ala	Arg	Asn	Arg	Ile	Asp	Thr	Phe	
			100							105						
ttc	ggg	acc	gcg	agc	ttc	tgc	gac	gar	cac	acc	atc	gag	gtc	gtc	cac	384
Phe	Gly	Thr	Ala	Ser	Phe	Cys	Asp	Glu	His	Thr	Ile	Glu	Val	Val	His	
			115							120						
ctc	aac	ggt	atg	gtg	gaa	acg	ctg	gtg	gcc	aag	cag	ttc	gtc	atc	gcc	432
Leu	Asn	Gly	Met	Val	Glu	Thr	Leu	Val	Ala	Lys	Gln	Phe	Val	Ile	Ala	
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acc	ggg	tcg	cgt	ccc	tac	cgc	ccg	gcc	gat	gtc	gat	ttc	acc	cat	ccg	480
Thr	Gly	Ser	Arg	Pro	Tyr	Arg	Pro	Ala	Asp	Val	Asp	Phe	Thr	His	Pro	
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cgc	atc	tac	gac	agc	gac	acc	atc	ctc	agc	ctc	ggc	cac	acg	ccg	cgc	528
Arg	Ile	Tyr	Asp	Ser	Asp	Thr	Ile	Leu	Ser	Leu	Gly	His	Thr	Pro	Arg	
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cgg	ttg	atc	atc	tac	gga	gcg	ggg	gtg	atc	ggc	tgc	gaa	tat	gcc	tcc	576
Arg	Leu	Ile	Ile	Tyr	Gly	Ala	Gly	Val	Ile	Gly	Cys	Glu	Tyr	Ala	Ser	
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atc	ttc	agt	ggg	ctg	ggc	gtg	ctg	gtc	gac	ctc	atc	gac	aac	cgc	gac	624
Ile	Phe	Ser	Gly	Leu	Gly	Val	Leu	Val	Asp	Leu	Ile	Asp	Asn	Arg	Asp	
			195							200						
caa	ctg	ctc	agt	ttc	ctc	gac	gac	gaa	atc	tcc	gac	tcg	ctc	agc	tac	672
Gln	Leu	Leu	Ser	Phe	Leu	Asp	Asp	Glu	Ile	Ser	Asp	Ser	Leu	Ser	Tyr	
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cac	ctg	cgc	aac	aac	aac	gtg	ctg	atc	cgc	cac	aac	gar	gaa	tac	gag	720
His	Leu	Arg	Asn	Asn	Asn	Val	Leu	Ile	Arg	His	Asn	Glu	Glu	Tyr	Glu	
			225							230						
cgt	gtc	gaa	ggc	ctg	gac	aac	ggg	gtg	atc	ctg	cac	ctc	aag	tcc	ggc	768
Arg	Val	Glu	Gly	Leu	Asp	Asn	Gly	Val	Ile	Leu	His	Leu	Lys	Ser	Gly	
			245							250						
aag	aag	atc	aag	gcc	gac	gcc	ttc	ctg	tgg	agc	aac	ggc	cgt	acc	ggc	816
Lys	Lys	Ile	Lys	Ala	Asp	Ala	Phe	Leu	Trp	Ser	Asn	Gly	Arg	Thr	Gly	
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aat	acc	gac	aag	ctg	ggc	ctg	gag	aac	atc	ggt	ctc	aag	gcc	aat	ggt	864
Asn	Thr	Asp	Lys	Leu	Gly	Leu	Glu	Asn	Ile	Gly	Leu	Lys	Ala	Asn	Gly	
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cgc	gga	cag	atc	cag	gtc	gac	gag	cac	tac	cgt	acc	gaa	gtc	agc	aam	912
Arg	Gly	Gln	Ile	Gln	Val	Asp	Glu	His	Tyr	Arg	Thr	Glu	Val	Ser	Xaa	
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Ile	Tyr	Ala	Ala	Gly	Asp	Val	Ile	Gly	Trp	Pro	Ser	Leu	Ala	Ser	Ala	
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gcc tat gac cag ggt cgt tcg gcc gcg ggc agt atc acc gag aac gat 1008
Ala Tyr Asp Gln Gly Arg Ser Ala Ala Gly Ser Ile Thr Glu Asn Asp
          325                      330                      335

agc tgg cgt ttc gtc gac gac gtg ccg acc ggc atc tac acc att ccg 1056
Ser Trp Arg Phe Val Asp Asp Val Pro Thr Gly Ile Tyr Thr Ile Pro
          340                      345                      350

gag atc agt tcg gtc ggc aag acc gag cgc gaa ctg acc cag gcg aag 1104
Glu Ile Ser Ser Val Gly Lys Thr Glu Arg Glu Leu Thr Gln Ala Lys
          355                      360                      365

gtt ccc tac gag gtc ggc aag gcc ttc ttc aag ggc atg gcc cgg gca 1152
Val Pro Tyr Glu Val Gly Lys Ala Phe Phe Lys Gly Met Ala Arg Ala
          370                      375                      380

cag atc gcc gtc gag aag gcc ggc atg ctg aag atc ctc ttt cac cgc 1200
Gln Ile Ala Val Glu Lys Ala Gly Met Leu Lys Ile Leu Phe His Arg
          385                      390                      395                      400

gag acg ctg gaa atc ctc ggc gtg cac tgc ttc ggc tat cag gct tcg 1248
Glu Thr Leu Glu Ile Leu Gly Val His Cys Phe Gly Tyr Gln Ala Ser
          405                      410                      415

gaa atc gtc cat atc ggc cag gcg atc atg aac cag aag ggc gag gcg 1296
Glu Ile Val His Ile Gly Gln Ala Ile Met Asn Gln Lys Gly Glu Ala
          420                      425                      430

aat acc ctc aag tat ttc atc aac acc acc ttc aac tac ccg acc atg 1344
Asn Thr Leu Lys Tyr Phe Ile Asn Thr Thr Phe Asn Tyr Pro Thr Met
          435                      440                      445

gcc gag gcc tac cgg gtg gcg gcc tac gac ggt ctc aat cgg ctt ttt 1392
Ala Glu Ala Tyr Arg Val Ala Ala Tyr Asp Gly Leu Asn Arg Leu Phe
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tga 1395

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<210> 2

<211> 464

<212> PRT

<213> Azotobacter vinelandii

<220>

<221> misc\_feature

<222> (5)..(5)

<223> The 'Xaa' at location 5 stands for Lys, or Asn.

<220>

<221> misc\_feature

<222> (304)..(304)

<223> The 'Xaa' at location 304 stands for Lys, or Asn.

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Met Ala Val Tyr Xaa Tyr Asp Val Val Val Ile Gly Thr Gly Pro Ala

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5

10

15



Gly Glu Gly Ala Ala Met Asn Ala Val Lys Ala Gly Arg Lys Val Ala  
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Val Val Asp Asp Arg Pro Gln Val Gly Gly Asn Cys Thr His Leu Gly  
35 40 45

Thr Ile Pro Ser Lys Ala Leu Arg His Ser Val Arg Gln Ile Met Gln  
50 55 60

Tyr Asn Asn Asn Pro Leu Phe Arg Gln Ile Gly Glu Pro Arg Trp Phe  
65 70 75 80

Ser Phe Ala Asp Val Leu Lys Ser Ala Glu Gln Val Ile Ala Lys Gln  
85 90 95

Val Ser Ser Arg Thr Gly Tyr Tyr Ala Arg Asn Arg Ile Asp Thr Phe  
100 105 110

Phe Gly Thr Ala Ser Phe Cys Asp Glu His Thr Ile Glu Val Val His  
115 120 125

Leu Asn Gly Met Val Glu Thr Leu Val Ala Lys Gln Phe Val Ile Ala  
130 135 140

Thr Gly Ser Arg Pro Tyr Arg Pro Ala Asp Val Asp Phe Thr His Pro  
145 150 155 160

Arg Ile Tyr Asp Ser Asp Thr Ile Leu Ser Leu Gly His Thr Pro Arg  
165 170 175

Arg Leu Ile Ile Tyr Gly Ala Gly Val Ile Gly Cys Glu Tyr Ala Ser  
180 185 190

Ile Phe Ser Gly Leu Gly Val Leu Val Asp Leu Ile Asp Asn Arg Asp  
195 200 205

Gln Leu Leu Ser Phe Leu Asp Asp Glu Ile Ser Asp Ser Leu Ser Tyr  
210 215 220

His Leu Arg Asn Asn Asn Val Leu Ile Arg His Asn Glu Glu Tyr Glu  
225 230 235 240

Arg Val Glu Gly Leu Asp Asn Gly Val Ile Leu His Leu Lys Ser Gly  
245 250 255

Lys Lys Ile Lys Ala Asp Ala Phe Leu Trp Ser Asn Gly Arg Thr Gly  
260 265 270

Asn Thr Asp Lys Leu Gly Leu Glu Asn Ile Gly Leu Lys Ala Asn Gly  
275 280 285 )

Arg Gly Gln Ile Gln Val Asp Glu His Tyr Arg Thr Glu Val Ser Xaa  
290 295 300

Ile Tyr Ala Ala Gly Asp Val Ile Gly Trp Pro Ser Leu Ala Ser Ala  
305 310 315 320

Ala Tyr Asp Gln Gly Arg Ser Ala Ala Gly Ser Ile Thr Glu Asn Asp  
                   325                  330                  335

Ser Trp Arg Phe Val Asp Asp Val Pro Thr Gly Ile Tyr Thr Ile Pro  
                   340                  345                  350

Glu Ile Ser Ser Val Gly Lys Thr Glu Arg Glu Leu Thr Gln Ala Lys  
                   355                  360                  365

Val Pro Tyr Glu Val Gly Lys Ala Phe Phe Lys Gly Met Ala Arg Ala  
                   370                  375                  380

Gln Ile Ala Val Glu Lys Ala Gly Met Leu Lys Ile Leu Phe His Arg  
                   385                  390                  395                  400

Glu Thr Leu Glu Ile Leu Gly Val His Cys Phe Gly Tyr Gln Ala Ser  
                   405                  410                  415

Glu Ile Val His Ile Gly Gln Ala Ile Met Asn Gln Lys Gly Glu Ala  
                   420                  425                  430

Asn Thr Leu Lys Tyr Phe Ile Asn Thr Thr Phe Asn Tyr Pro Thr Met  
                   435                  440                  445

Ala Glu Ala Tyr Arg Val Ala Ala Tyr Asp Gly Leu Asn Arg Leu Phe  
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<210> 3  
 <211> 52  
 <212> DNA  
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 <211> 71  
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 gcaactaaaa a 71

<210> 5  
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 <212> DNA  
 <213> Artificial Sequence

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$\langle 211 \rangle$	38

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ENTERED

PCT09

## RAW SEQUENCE LISTING

PATENT APPLICATION: US/09/743,414A

DATE: 01/31/2003

TIME: 11:52:10

Input Set : A:\NIELSEN4.txt

Output Set: N:\CRF4\01312003\I743414A.raw

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4     Nissen, Torben L
5     Kielland-Brandt, Morten C
7 <120> TITLE OF INVENTION: Metabolically engineered microbial cell with an altered
8     metabolite production
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12 <140> CURRENT APPLICATION NUMBER: 09/743,414A
13 <141> CURRENT FILING DATE: 2001-01-10
15 <150> PRIOR APPLICATION NUMBER: PCT/DK99/00397
16 <151> PRIOR FILING DATE: 1999-07-12
18 <150> PRIOR APPLICATION NUMBER: PA 1998 00967
19 <151> PRIOR FILING DATE: 1998-07-10
21 <160> NUMBER OF SEQ ID NOS: 14
23 <170> SOFTWARE: PatentIn Ver. 2.1
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26 <211> LENGTH: 1395
27 <212> TYPE: DNA
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32 <222> LOCATION: (1)..(1395)
33 <223> OTHER INFORMATION:
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40 ggc gaa ggg gca gcg atg aat gcc gtg aag gcc ggg cgc aag gta gcg 96
41 Gly Glu Gly Ala Ala Met Asn Ala Val Lys Ala Gly Arg Lys Val Ala
42 20 25 30
44 gtc gtg gat gat cgc ccc cag gtc ggc ggc aac tgc acc cac ctc gga 144
45 Val Val Asp Asp Arg Pro Gln Val Gly Gly Asn Cys Thr His Leu Gly
46 35 40 45
48 acc att ccc tcc aag gcg ctg cgc cac tcg gtg cgg cag atc atg cag 192
49 Thr Ile Pro Ser Lys Ala Leu Arg His Ser Val Arg Gln Ile Met Gln
50 50 55 60
52 tac aac aac aat ccg ctg ttc cgc cag atc ggc gag ccg cgc tgg ttt 240
53 Tyr Asn Asn Asn Pro Leu Phe Arg Gln Ile Gly Glu Pro Arg Trp Phe
54 65 70 75 80
56 tcc ttc gcc gat gtc ctg aag agc gcc gag cag gtc atc gcc aag cag 288
57 Ser Phe Ala Asp Val Leu Lys Ser Ala Glu Gln Val Ile Ala Lys Gln
58 85 90 95
60 gtt tcc tcg cgg acc ggc tac tat gcg cgc aac cgt atc gat acc ttc 336
61 Val Ser Ser Arg Thr Gly Tyr Tyr Ala Arg Asn Arg Ile Asp Thr Phe

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DATE: 01/31/2003

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66          115          120          125
68 ctc aac ggt atg gtg gaa acg ctg gtg gcc aag cag ttc gtc atc gcc 432
69 Leu Asn Gly Met Val Glu Thr Leu Val Ala Lys Gln Phe Val Ile Ala
70          130          135          140
72 acc ggg tcg cgt ccc tac cgc ccg gcc gat gtc gat ttc acc cat ccg 480
73 Thr Gly Ser Arg Pro Tyr Arg Pro Ala Asp Val Asp Phe Thr His Pro
74 145          150          155          160
76 cgc atc tac gac agc gac acc atc ctc agc ctc ggc cac acg ccg cgc 528
77 Arg Ile Tyr Asp Ser Asp Thr Ile Leu Ser Leu Gly His Thr Pro Arg
78          165          170          175
80 cgg ttg atc atc tac gga gcg ggg gtg atc ggc tgc gaa tat gcc tcc 576
81 Arg Leu Ile Ile Tyr Gly Ala Gly Val Ile Gly Cys Glu Tyr Ala Ser
82          180          185          190
84 atc ttc agt ggg ctg ggc gtg ctg gtc gac ctc atc gac aac cgc gac 624
85 Ile Phe Ser Gly Leu Gly Val Leu Val Asp Leu Ile Asp Asn Arg Asp
86          195          200          205
88 caa ctg ctc agt ttc ctc gac gac gaa atc tcc gac tcg ctc agc tac 672
89 Gln Leu Leu Ser Phe Leu Asp Asp Glu Ile Ser Asp Ser Leu Ser Tyr
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93 His Leu Arg Asn Asn Asn Val Leu Ile Arg His Asn Glu Glu Tyr Glu
94 225          230          235          240
96 cgt gtc gaa ggc ctg gac aac ggg gtg atc ctg cac ctc aag tcc ggc 768
97 Arg Val Glu Gly Leu Asp Asn Gly Val Ile Leu His Leu Lys Ser Gly
98          245          250          255
100 aag aag atc aag gcc gac gcc ttc ctg tgg agc aac ggc cgt acc gcc 816
101 Lys Lys Ile Lys Ala Asp Ala Phe Leu Trp Ser Asn Gly Arg Thr Gly
102          260          265          270
104 aat acc gac aag ctg ggc ctg gag aac atc ggt ctc aag gcc aat ggt 864
105 Asn Thr Asp Lys Leu Gly Leu Glu Asn Ile Gly Leu Lys Ala Asn Gly
106          275          280          285
108 cgc gga cag atc cag gtc gac gag cac tac cgt acc gaa gtc agc aam 912
W--> 109 Arg Gly Gln Ile Gln Val Asp Glu His Tyr Arg Thr Glu Val Ser Xaa
110          290          295          300
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113 Ile Tyr Ala Ala Gly Asp Val Ile Gly Trp Pro Ser Leu Ala Ser Ala
114 305          310          315          320
116 gcc tat gac cag ggt cgt tcg gcc gcg ggc agt atc acc gag aac gat 1008
117 Ala Tyr Asp Gln Gly Arg Ser Ala Ala Gly Ser Ile Thr Glu Asn Asp
118          325          330          335
120 agc tgg cgt ttc gtc gac gac gtg ccg acc ggc atc tac acc att ccg 1056
121 Ser Trp Arg Phe Val Asp Asp Val Pro Thr Gly Ile Tyr Thr Ile Pro
122          340          345          350
124 gag atc agt tcg gtc ggc aag acc gag cgc gaa ctg acc cag gcg aag 1104
125 Glu Ile Ser Ser Val Gly Lys Thr Glu Arg Glu Leu Thr Gln Ala Lys
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DATE: 01/31/2003

PATENT APPLICATION: US/09/743,414A

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Output Set: N:\CRF4\01312003\I743414A.raw

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128 gtt ccc tac gag gtc ggc aag gcc ttc ttc aag ggc atg gcc cgg gca 1152
129 Val Pro Tyr Glu Val Gly Lys Ala Phe Phe Lys Gly Met Ala Arg Ala
130 370 375 380
132 cag atc gcc gtc gag aag gcc ggc atg ctg aag atc ctc ttt cac cgc 1200
133 Gln Ile Ala Val Glu Lys Ala Gly Met Leu Lys Ile Leu Phe His Arg
134 385 390 395 400
136 gag acg ctg gaa atc ctg ggc gtg cac tgc ttc ggc tat cag gct tcg 1248
137 Glu Thr Leu Glu Ile Leu Gly Val His Cys Phe Gly Tyr Gln Ala Ser
138 405 410 415
140 gaa atc gtc cat atc ggc cag gcg atc atg aac cag aag ggc gag gcg 1296
141 Glu Ile Val His Ile Gly Gln Ala Ile Met Asn Gln Lys Gly Glu Ala
142 420 425 430
144 aat acc ctc aag tat ttc atc aac acc acc ttc aac tac ccg acc atg 1344
145 Asn Thr Leu Lys Tyr Phe Ile Asn Thr Thr Phe Asn Tyr Pro Thr Met
146 435 440 445
148 gcc gag gcc tac cgg gtg gcg gcc tac gac ggt ctc aat cgg ctt ttt 1392
149 Ala Glu Ala Tyr Arg Val Ala Ala Tyr Asp Gly Leu Asn Arg Leu Phe
150 450 455 460
152 tga 1395
156 <210> SEQ ID NO: 2
157 <211> LENGTH: 464
158 <212> TYPE: PRT
159 <213> ORGANISM: Azotobacter vinelandii
161 <220> FEATURE:
162 <221> NAME/KEY: misc_feature
163 <222> LOCATION: (5)..(5)
164 <223> OTHER INFORMATION: The 'Xaa' at location 5 stands for Lys, or Asn.
166 <220> FEATURE:
167 <221> NAME/KEY: misc_feature
168 <222> LOCATION: (304)..(304)
169 <223> OTHER INFORMATION: The 'Xaa' at location 304 stands for Lys, or Asn.
171 <400> SEQUENCE: 2
W--> 172 Met Ala Val Tyr Xaa Tyr Asp Val Val Val Ile Gly Thr Gly Pro Ala
173 1 5 10 15
175 Gly Glu Gly Ala Ala Met Asn Ala Val Lys Ala Gly Arg Lys Val Ala
176 20 25 30
178 Val Val Asp Asp Arg Pro Gln Val Gly Gly Asn Cys Thr His Leu Gly
179 35 40 45
181 Thr Ile Pro Ser Lys Ala Leu Arg His Ser Val Arg Gln Ile Met Gln
182 50 55 60
184 Tyr Asn Asn Asn Pro Leu Phe Arg Gln Ile Gly Glu Pro Arg Trp Phe
185 65 70 75 80
187 Ser Phe Ala Asp Val Leu Lys Ser Ala Glu Gln Val Ile Ala Lys Gln
188 85 90 95
190 Val Ser Ser Arg Thr Gly Tyr Tyr Ala Arg Asn Arg Ile Asp Thr Phe
191 100 105 110
193 Phe Gly Thr Ala Ser Phe Cys Asp Glu His Thr Ile Glu Val Val His
194 115 120 125
196 Leu Asn Gly Met Val Glu Thr Leu Val Ala Lys Gln Phe Val Ile Ala

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RAW SEQUENCE LISTING

PATENT APPLICATION: US/09/743,414A

DATE: 01/31/2003

TIME: 11:52:10

Input Set : A:\NIELSEN4.txt

Output Set: N:\CRF4\01312003\I743414A.raw

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197      130      135      140
199 Thr Gly Ser Arg Pro Tyr Arg Pro Ala Asp Val Asp Phe Thr His Pro
200 145      150      155      160
202 Arg Ile Tyr Asp Ser Asp Thr Ile Leu Ser Leu Gly His Thr Pro Arg
203      165      170      175
205 Arg Leu Ile Ile Tyr Gly Ala Gly Val Ile Gly Cys Glu Tyr Ala Ser
206      180      185      190
208 Ile Phe Ser Gly Leu Gly Val Leu Val Asp Leu Ile Asp Asn Arg Asp
209      195      200      205
211 Gln Leu Leu Ser Phe Leu Asp Asp Glu Ile Ser Asp Ser Leu Ser Tyr
212      210      215      220
214 His Leu Arg Asn Asn Asn Val Leu Ile Arg His Asn Glu Glu Tyr Glu
215 225      230      235      240
217 Arg Val Glu Gly Leu Asp Asn Gly Val Ile Leu His Leu Lys Ser Gly
218      245      250      255
220 Lys Lys Ile Lys Ala Asp Ala Phe Leu Trp Ser Asn Gly Arg Thr Gly
221      260      265      270
223 Asn Thr Asp Lys Leu Gly Leu Glu Asn Ile Gly Leu Lys Ala Asn Gly
224      275      280      285
W--> 226 Arg Gly Gln Ile Gln Val Asp Glu His Tyr Arg Thr Glu Val Ser Xaa
227      290      295      300
229 Ile Tyr Ala Ala Gly Asp Val Ile Gly Trp Pro Ser Leu Ala Ser Ala
230 305      310      315      320
232 Ala Tyr Asp Gln Gly Arg Ser Ala Ala Gly Ser Ile Thr Glu Asn Asp
233      325      330      335
235 Ser Trp Arg Phe Val Asp Asp Val Pro Thr Gly Ile Tyr Thr Ile Pro
236      340      345      350
238 Glu Ile Ser Ser Val Gly Lys Thr Glu Arg Glu Leu Thr Gln Ala Lys
239      355      360      365
241 Val Pro Tyr Glu Val Gly Lys Ala Phe Phe Lys Gly Met Ala Arg Ala
242      370      375      380
244 Gln Ile Ala Val Glu Lys Ala Gly Met Leu Lys Ile Leu Phe His Arg
245 385      390      395      400
247 Glu Thr Leu Glu Ile Leu Gly Val His Cys Phe Gly Tyr Gln Ala Ser
248      405      410      415
250 Glu Ile Val His Ile Gly Gln Ala Ile Met Asn Gln Lys Gly Glu Ala
251      420      425      430
253 Asn Thr Leu Lys Tyr Phe Ile Asn Thr Thr Phe Asn Tyr Pro Thr Met
254      435      440      445
256 Ala Glu Ala Tyr Arg Val Ala Ala Tyr Asp Gly Leu Asn Arg Leu Phe
257      450      455      460
261 <210> SEQ ID NO: 3
262 <211> LENGTH: 52
263 <212> TYPE: DNA
264 <213> ORGANISM: Artificial Sequence
266 <220> FEATURE:
267 <223> OTHER INFORMATION: Description of Artificial Sequence: Primer
269 <400> SEQUENCE: 3
270 attcatcgat gaattctatc ttatggtoce attctttact gcactgttta ca 52

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09/743414  
526 Rec'd PCT/PTO 10 JAN 2001

WO 00/03020

PCT/DK99/00397

SEQUENCE LISTING

<110> Nielsen, Jens  
Nissen, Torben L

<120> Metabolically engineered microbial cell with an altered  
metabolite production

<130> 29944/JL

<140>

<141>

<150> PA 1998 00967

<151> 1998-07-10

<160> 14

<170> PatentIn Ver. 2.1

<210> 1

<211> 1395

<212> DNA

<213> Azotobacter vinelandii

<220>

<221> CDS

<222> (1)..(1395)

<223> s at pos 15 is c or g; Xaa is Asn or Lys;  
r at pos 363 is a or g; r at pos 711 is a or g;  
m at pos 912 is a or c;  
Xaa is Asn or Lys

<400> 1

atg gct gta tat aas tac gat gtg gtg gta atc ggc aca ggc cct gct 48  
Met Ala Val Tyr Xaa Tyr Asp Val Val Val Ile Gly Thr Gly Pro Ala

1 5 10 15

ggc gaa ggg gca gcg atg aat gcc gtg aag gcc ggg cgc aag gta gcg 96  
Gly Glu Gly Ala Ala Met Asn Ala Val Lys Ala Gly Arg Lys Val Ala

20 25 30

gtc gtg gat gat cgc ccc cag gtc ggc ggc aac tgc acc cac ctc gga 144  
Val Val Asp Asp Arg Pro Gln Val Gly Gly Asn Cys Thr His Leu Gly

35 40 45

acc att ccc tcc aag gcg ctg cgc cac tcg gtg cgg cag atc atg cag 192  
Thr Ile Pro Ser Lys Ala Leu Arg His Ser Val Arg Gln Ile Met Gln



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245	250	255	
aag aag atc aag gcc gac gcc ttc ctg tgg agc aac ggc cgt acc ggc			816
Lys Lys Ile Lys Ala Asp Ala Phe Leu Trp Ser Asn Gly Arg Thr Gly			
260	265	270	
aat acc gac aag ctg ggc ctg gag aac atc ggt ctc aag gcc aat ggt			864
Asn Thr Asp Lys Leu Gly Leu Glu Asn Ile Gly Leu Lys Ala Asn Gly			
275	280	285	
cgc gga cag atc cag gtc gac gag cac tac cgt acc gaa gtc agc aam			912
Arg Gly Gln Ile Gln Val Asp Glu His Tyr Arg Thr Glu Val Ser Xaa			
290	295	300	
att tat gcc gct ggt gac gtg atc ggc tgg ccg agc ctg gcc agc gcc			960
Ile Tyr Ala Ala Gly Asp Val Ile Gly Trp Pro Ser Leu Ala Ser Ala			
305	310	315	320
gcc tat gac cag ggt cgt tcg gcc gcg ggc agt atc acc gag aac gat			1008
Ala Tyr Asp Gln Gly Arg Ser Ala Ala Gly Ser Ile Thr Glu Asn Asp			
325	330	335	
agc tgg cgt ttc gtc gac gac gtg ccg acc ggc atc tac acc att ccg			1056
Ser Trp Arg Phe Val Asp Asp Val Pro Thr Gly Ile Tyr Thr Ile Pro			
340	345	350	
gag atc agt tcg gtc ggc aag acc gag cgc gaa ctg acc cag gcg aag			1104
Glu Ile Ser Ser Val Gly Lys Thr Glu Arg Glu Leu Thr Gln Ala Lys			
355	360	365	
gtt ccc tac gag gtc ggc aag gcc ttc ttc aag ggc atg gcc cgg gca			1152
Val Pro Tyr Glu Val Gly Lys Ala Phe Phe Lys Gly Met Ala Arg Ala			
370	375	380	
cag atc gcc gtc gag aag gcc ggc atg ctg aag atc ctc ttt cac cgc			1200
Gln Ile Ala Val Glu Lys Ala Gly Met Leu Lys Ile Leu Phe His Arg			
385	390	395	400
gag acg ctg gaa atc ctc ggc gtg cac tgc ttc ggc tat cag gct tcg			1248
Glu Thr Leu Glu Ile Leu Gly Val His Cys Phe Gly Tyr Gln Ala Ser			
405	410	415	
gaa atc gtc cat atc ggc cag gcg atc atg aac cag aag ggc gag gcg			1296
Glu Ile Val His Ile Gly Gln Ala Ile Met Asn Gln Lys Gly Glu Ala			
420	425	430	
aat acc ctc aag tat ttc atc aac acc acc ttc aac tac ccg acc atg			1344
Asn Thr Leu Lys Tyr Phe Ile Asn Thr Thr Phe Asn Tyr Pro Thr Met			





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420

425

430

Asn Thr Leu Lys Tyr Phe Ile Asn Thr Thr Phe Asn Tyr Pro Thr Met  
435 440 445

Ala Glu Ala Tyr Arg Val Ala Ala Tyr Asp Gly Leu Asn Arg Leu Phe  
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<210> 3

<211> 52

<212> DNA

<213> Artificial Sequence

<220>

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<211> 71

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Primer

<400> 4

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<210> 5

<211> 24

<212> DNA

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<223> Description of Artificial Sequence: Primer

<400> 5

ggttttctac aatctccaaa agag 24

<210> 6

<211> 38

<212> DNA

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<220>

<223> Description of Artificial Sequence: Primer

<400> 6

gcgcgagatc ttctagaatg ctttttgata acaaaaat

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<210> 7

<211> 38

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Primer

<400> 7

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38

<210> 8

<211> 19

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Primer

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19

<210> 9

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

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38

<210> 10

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<211> 38

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<210> 11

<211> 38

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Primer

<400> 11

gcgcgggtatc ctctagaatg gctgaagcaa gcatcgaa

38

<210> 12

<211> 38

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Primer

<400> 12

cgcgcggtatc cccgcgggta tgaagattct ctttcaaa

38

<210> 13

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 13

tacgaagatc tgctgtatat aactacgatg tgggtg

36

<210> 14



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<212> DNA

<220>

<400> 14

32